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IGF SYSTEM, CD99 AND TUMOR-SPECIFIC
CHROMOSOMAL TRANSLOCATIONS: EVALUATION OF
THEIR CROSS-TALK AND DEFINITION OF THEIR ROLE
IN EWING SARCOMA AND PROSTATE CANCER

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Introduction

1. Tumor-specific chromosomal translocations

1.1 General aspects

Cancer is due to acquired genetic changes, sometimes associated with inherited predisposing mutations. Hypothesis that chromosomal changes are responsible for neoplasia was first proposed by Theodor Boveri in 1914 [1] but it was completely accepted with improvements in cytogenetic and molecular techniques. Translocations together with deletions and inversions represent the three main cytogenetic changes in cancer. Translocations can be divided in specific, when consistently found in a certain tumor types, and idiopathic, when observed in the tumor from one patient [2]. Up until recently, importance of tumor-specific translocations was mainly diagnostic but the more recent characterization of these rearrangements has provided important insights into the neoplastic process [3]. Consequences of chromosomal translocations are potentially two: activation of genes located at or near the breakpoints that are pivotal in the control of cell growth and differentiation or generation of a chimaeric gene resulting from fusion between two unrelated genes positioned at each of the involved chromosomal breakpoints. In both of the cases, a factor with altered expression or function is produced and it cooperates in establishing a transformed phenotype. In some cases, translocations can inactivate oncosuppressor genes such as repression of TEL1 as a consequence of the TEL1-AML translocation [4]. The first described translocation was the Philadelphia chromosome, a reciprocal chromosomal translocation involving chromosomes 9 and 22 in chronic myelogenous leukemia (CML) [5]. Subsequently, a translocation between chromosomes 8 and 14 was discovered in Burkitt’s lymphoma [6] where c-MYC oncogene translocates to the immunoglobulin heavy chain loci on chromosome 14 [7]. These discoveries stimulated interest in cancer cytogenetics and, as a consequence, information on chromosomal aberrations in cancer has increased over the past two decades. In the early 1990s, specific translocations were found in sarcomas including Ewing sarcoma, myxoid liposarcoma and synovial sarcomas. Until that period, it was believed that chromosomal translocations were restricted to lymphoid cancers and few sarcomas but subsequent studies showed that carcinomas can express chromosomal translocations. The first example was the fusion between RET gene
encoding a tyrosine kinase receptor with the CCDC6 gene in papillary thyroid carcinoma [8]. Afterwards, other gene fusions have been discovered in carcinomas. Particularly, important fusion genes have been recently identified in prostate and lung cancer.

Molecular cytogenetic techniques including fluorescence in situ hybridization (FISH), multicolour FISH and array-based comparative genomic hybridization dramatically improved analysis of chromosomal breakpoints that now can be mapped very precisely [9]. Cytogenetic characterization allowed identification of almost 337 genes involved in fusions in neoplastic disorders and they represent a substantial proportion of all mutated genes implicated in oncogenesis [9]. Many of these chromosomal translocations are associated with distinct tumor types, clinical features and characteristic gene expression profiles. This information has become an important tool in the management of cancer patients helping to establish a correct diagnosis, select appropriate treatment, and predict outcome [9]. As a consequence, fusion genes represent useful biomarkers which identification represents a remarkable advantage in a certain tumor type.

1.2 Gene fusions in haematological disorders

Leukaemias and lymphomas, together constituting 8% of all cancers, harbor translocations in almost all cases, representing 75% of all gene fusions known in human neoplasia. These high percentages lead to opinion that these disorders are exclusively caused by fusion genes. Actually, prevalence of most individual gene fusions is very low and few well-known specific changes are observed in 100% of cases and represent exceptions: BCR-ABL in CML, IGH-CCND1 in mantle cell lymphoma, MYC deregulation in Burkitt’s lymphoma and PML-RARA in acute promyelocytic leukaemia (APL). CML is characterized by a translocation between ABL gene on chromosome 9 and BCR gene promoter on chromosome 22. This results in the formation of a unique in-frame fusion mRNA and a constitutively activated protein tyrosine kinase that was shown to be oncogenic [10]. Subsequently, the tyrosine kinase inhibitor imatinib mesylate, specific for the ABL, was developed [11] and preclinical and clinical trials demonstrated its high efficacy becoming a new standard treatment for CML patients [12]. Burkitt’s lymphoma is a B cell tumor containing the t(8;14) translocation in 70% of cases and resulting in over-expression of c-MYC. Several therapeutic approaches have been developed to target MYC-dependent tumors including inhibitors of transcriptional machinery, inhibitors of MYC dimerization which prevents its DNA binding, blockade of MYC stability but clinical trials evaluating effectiveness in
lymphoid malignancies are lacking [13]. Chromosomal translocation involving chromosomes 11 and 14 in mantle cell lymphoma results in constitutional over-expression of cyclin D1 and, consequently, cell cycle deregulation. Nowadays, detection of t(11;14) or cyclin D1 over-expression represent crucial features for a correct diagnosis [14]. In over 98% of APL patients, a specific chromosomal translocation fuses PML gene on chromosome 15 to the RARA gene on chromosome 17 resulting in PML/RARA fusion protein which is the molecular determinant of the disease [15] and a useful tool for tumor diagnosis [16].

1.3 Gene fusions in sarcomas

Bone and soft tissue sarcomas represent a clinically and morphologically heterogeneous group of neoplasms of mesenchymal or neuroectodermal origin. The overall fraction of sarcomas with chimeric genes is 15 to 20%. Between sarcomas presenting chromosomal translocations, Ewing sarcoma, myxoid liposarcomas and synovial sarcomas harbor chimeric genes in 100% of cases [9]. Ewing sarcoma specific translocation involves EWSR1 gene on chromosome 22 and one of the ETS transcription factor genes, particularly FLI1, located on chromosome 11, in 85% of cases, or ERG, on chromosome 21, in 11% of cases. EWS-ETS fusion gene results in a chimeric protein with aberrant transcriptional activity. Since its discovery, EWS-FLI1 represented a fundamental tool for Ewing sarcoma diagnosis and a key element for a better understanding of tumor biology. Several studies demonstrated its importance in induction of a transformed phenotype mainly through identification of its target genes. Being exclusively located within the tumor and driving cell transformation, EWS-FLI1 represents an excellent therapeutic target and some compounds were developed in the past years in order to specifically block it. Particularly, the small molecule YK-4-279, disrupting interaction between EWS-FLI1 and RNA Helicase A (RHA), which is a critical mechanism for EWS transformation [17], was shown to induce apoptotic cell death [18] in preclinical studies but it never entered the clinic. Currently, additional preclinical studies are ongoing testing its efficacy alone or in combination with other compounds with the purpose to optimize its use [19-21]. Myxoid liposarcoma is characterized by a translocation fusing FUS gene, on chromosome 12, and CHOP gene on chromosome 16. The fusion FUS-CHOP protein functions as abnormal transcription factor and its relevance in the pathogenesis of myxoid liposarcoma is well established [22]. Recent evidences demonstrated that myxoid liposarcoma is particularly sensitive to Trabectedin (ET-734, Yondelis), a marine alkaloid which is cytotoxic against a variety of tumor cell
lines [23] and human tumor xenografts in vivo [24]. In vitro studies demonstrated that the high sensitivity of myxoid liposarcoma to this agent is due to the ability of Trabectedin to act as a differentiating agent by blocking the transactivating capability of FUS-CHOP [25, 26]. Clinical trials confirmed the high efficacy of Trabectedin in patients [27]. The molecular hallmark of synovial sarcoma is a pathognomonic reciprocal translocation leading to the fusion of SS18 to one of the homologs SSX genes, generating oncogenic SS18-SSX fusion proteins with aberrant transcriptional activity [28]. The specific biological function and the mechanism of action of SS18-SSX remain to be defined but it has a crucial role in tumorigenesis and progression. No specific agent has been developed yet to directly inhibit SS18-SSX as it is part of regulatory active complexes but recently some studies have been focused on inhibition of SS18-SSX-mediated pathways such as Wnt/β-catenin signaling. Preliminary data demonstrate a good in vitro response to these agents [28].

1.4 Gene fusions in carcinomas

Occurrence of gene fusions in malignant epithelial tumors is generally rare and this lead to the commonly view that chromosomal translocations have a minor role in the pathogenesis of carcinomas [9]. Actually, carcinomas are characterized by gene rearrangements as well but limitations of available techniques and the individual rarity of these alterations made them more difficult to detect. Overall, almost all lymphomas harbor translocations, whereas only one-fourth of all sarcomas is reported to possess the same. Gene fusion have been recently discovered in carcinomas so that the exact percentage of carcinomas harboring fusion genes is not clear. The most remarkable recently discovered fusion genes in epithelial tumors include TMPRSS2-ERG in prostate cancer and EML4-ALK in lung cancer. Prostate cancer is the first common cancer associated with a high frequency to a gene fusion as almost 70% of cases present this rearrangement. Through bioinformatic tools, Tomlins et al. in 2005 noticed a strong over-expression of ETS transcription factor genes, ERG and ETV1, in prostate cancer specimens [29]. Subsequently, the genes were found to be fused with the 5′ part of the prostate-specific gene TMPRSS2 located on chromosome 21. The result is an androgen-regulated over-expression of ERG or ETV1 which can stimulate transcription of target genes for cell growth, invasion and metastases and promote cancer progression [30, 31]. At clinical level, TMPRSS2-ERG was found to improve detection of clinically significant prostate cancer when combined with other biomarkers evaluation such as
PCA3 or SPINK1 [32-34]. In addition, prognostic relevance of *TMPRSS2-ERG* was investigated in several epidemiological studies, giving controversial results. The restricted expression of *TMPRSS2-ERG* to cancer cell made it a suitable therapeutic target [35]. Currently, *TMPRSS2-ERG* inhibition *in vitro* has been demonstrated to inhibit tumor growth. Specific siRNA via liposomal nanovector have been proposed [36] representing a potential efficacious treatment with low toxicity for prostate cancer patients.

Fusion between the anaplastic lymphoma kinase gene (*ALK*) and the echinoderm microtubule-associated protein-like 4 gene (*EML4*) has been detected in a subset of non-small cell lung cancers (NSCLCs). The *EML4-ALK* fusion gene encodes a fusion protein retaining the kinase domain of ALK [37]. Oncogenic fusion genes have been detected in approximately 2-7% of NSCLC patients. *EML4-ALK* defines a molecular subset of patients with distinct clinical characteristics and with a relevant therapeutic option in crizotinib, a small molecule inhibitor of both ALK and c-MET, with high tolerability and robust antitumor activity as demonstrated by clinical trials. Nowadays, crizotinib is a new standard of care for patients with advanced, *EML2-ALK*-positive, NSCLC [38].

### 1.5 ETS-associated translocations

The ETS transcription factors family was established from the v-ets oncogene, discovered as part of the transforming fusion protein of E26 avian replication-defective retrovirus. The v-ets oncogene was found to be able to transform fibroblasts, myeloblasts, and erythroblasts *in vitro*. Today, the ETS family is known as one of the largest families of transcriptional regulators, with various functions and activities. In human, 27 ETS members have been identified and they are characterized by the ETS domain, a 85 amino acids conserved sequence with DNA-binding capability. In particular, this domain is composed of three alpha helices and a four-stranded, beta sheet recognizing a core GGAA/T sequence (Ets binding site). ETS factors act as positive or negative regulators of expression of genes that are involved in various biological processes including cell proliferation, differentiation, hematopoiesis, apoptosis, metastasis, tissue remodeling, angiogenesis and transformation [39]. The importance of ETS genes in cancer was early demonstrated by *in vitro* and *in vivo* studies showing cellular transformation induced by ETS1, ETS2 and ERG [40-42]. In addition, ETS relevance in cancer has been demonstrated by the observation that ETS genes are frequently mutated in tumors including frequent location at translocation
breakpoints. Particularly, ERG involvement in chromosomal rearrangements was described in leukaemias and solid tumors including sarcomas and carcinomas. In leukaemias, FUS gene on chromosome 16 was described to be fused with ERG in acute myeloid leukaemia [43, 44]. The product is the FUS-ERG fusion protein, an early event in cancer progression with established oncogenic properties including inhibition of differentiation into neutrophils of a mouse myeloid precursor cell line [45]. As previously mentioned, chromosomal rearrangements involving ERG have been also described in Ewing sarcoma, where the ETS generates fusion genes with EWS driving cell transformation, and prostate cancer, where the ETS genes fuse with androgen-related TMPRSS2. Considering ETS involvement in chromosomal translocations as a shared mechanism between different tumor types, identification of common or distinctive mechanisms sustained by ETS rearrangement that could be relevant for tumor biology and clinical management of tumors represents the objective of several studies, including the one here presented.

2. Ewing sarcoma

2.1 General characteristics

In 1921, Dr. James Ewing described for the first time a lesion that he named a “diffuse endothelioma of bone” [46] and that today is known as Ewing sarcoma (ES). ES belongs to the Ewing’s sarcoma family tumors (ESFT) comprehending osseous ES, extra-skeletal ES, Askin tumor and peripheral primitive neuroectodermal tumor (PNET) [47, 48]. ES is a rare highly aggressive and poor differentiated disease composed by small round cells. It mostly affects bone but can also involve soft tissues including kidney, lung, bladder, prostate. From a biological point of view, ESFT are characterized by a specific translocation resulting in most of the cases in the EWS-FLI1 chimeric transcription factor that is transforming in cells.

2.2 Epidemiology and risk

ES is the second most common tumor of bone after osteosarcoma among children and young adults, with an annual incidence rate of three per million. Some evidences show that boys are more commonly affected than girls with a ratio of 1.5:1. In 90% of the
cases, patients are between the ages of 5 and 20 years while is rare in individuals older than 40 and younger than 5 years. No environmental factor has been identified as a risk factor for this tumor [49] and there is no evidence regarding familiar predisposition [50] beside some studies reported an increased risk of neuroectodermal tumors and stomach cancer in family members of ES patients [51] or congenital mesenchymal defects in ES patients [52]. Ethnicity represents an important epidemiologic factor with the highest risk in Caucasians more than Africans and Asians. Some studies demonstrated an increased risk of secondary cancers after ES treatment including radiation-induced osteosarcoma or therapy-related acute myeloid leukemia but incidence of ES as a second tumor after therapy is rare [53, 54].

2.3 Localization, histopathology and staging

ES mainly arises as a primitive tumor of flat, short and long bones. In the appendicular skeleton, femur is the most common localization followed by tibia, humerus, fibula, and forearm bones. In the trunk, the most frequent localization is the pelvis, followed by vertebrae and sacrum, scapula, ribs, and clavicle. In long bones ES can arise from midshaft but may involve a larger portion or even the entire bone. Skull, hands and feet involvement in rare. More frequently, ES presents as a permeative, infiltrative, poorly defined osteolysis. The cortex is normally breached or destroyed by the tumor while rarely the tumor remains intramedullary. ES is very soft, grayish and can be hyperemic or hemorrhagic. In the center it is normally necrotic, with a semiliquid appearance. Microscopically, the tumor is composed of small round cells closely packed and without matrix. Cytoplasm is scarce, pale, granular, and clear to eosinophilic with poorly defined limits. Nuclei are round/oval, with a distinct nuclear membrane and powder-like chromatin, with one or more tiny nucleoli (Figure 1). Current staging of ES was proposed by Enneking: EW I, solitary intraosseous; EW II, solitary extraosseous; EW III, multicentric, skeletal; EW IV, distant metastases [55].
2.4 Course and diagnosis

Pain is the earliest symptom of ES together with swelling in a bone or joint. Systemic symptoms such as weight loss and fever can be associated but sometimes lead to erroneous diagnosis. ES usually displays an aggressive growth beside some cases where ES remains intraosseous have been described. Metastases from ES at presentation are about 20 to 25% and sites of metastases are the lungs in 50 percent of cases, bone in 25 percent of cases, and bone marrow in 25 percent of cases. Lymph nodes and brain can also be involved in metastases from ES [56]. In some cases, presence of multiple bone lesions at diagnosis makes difficult to distinguish between metastases and a multicentric origin of primitive tumor. The five-year survival rate is 60% in patients who present with primitive tumor while it decreases at 30% in patients with metastasis. The ten-year survival rate accounts 55% [57]. The poor prognosis of ES is due to both tumor aggressiveness, as it displays an elevated rate of recurrence and metastases, and secondary diseases caused by chemotherapy and radiotherapy [58]. Diagnosis of ES requires a multidisciplinary approach involving immunological, genetic and imaging techniques. Absence of exclusive morphologic characteristics of ES made the diagnosis of this tumor difficult up to the 90’s when EWS-FLI1 fusion gene was discovered and, consequently, the molecular analysis was introduced in the clinical practice. Biopsy from at least two sites of the tumor should be obtained for pathological, cytogenetic and molecular studies. In addition, as ES metastatizes to bone marrow, patients must undergo bone marrow aspiration and biopsy at two or more sites [56]. Plain radiographs and magnetic resonance imaging of the entire affected bone should be included in the evaluation.
The histogenesis of ES remains unknown despite the efforts and numerous studies performed in this field. Many groups proposed different origins of this tumor but the most accredited hypothesis are two: neural and mesenchymal histogenesis. The neural hypothesis arises from the observation that EWS-FLI1 rearrangement is a common feature between ES of bone and PNET. In addition, different studies evidenced that both osseous [59] and extra-osseous [60] ES cell lines undergo neural differentiation upon stimulation with differentiating agents. In particular, the study from Cavazzana et al. evidenced that, in five osseous ES cell lines, treatment with AMPc +/- NGF induced marked morphologic evidence of neural differentiation including neural filaments, synthesis of neuron-specific enzymes such as cholinesterases and NSE, and expression of neural tissue cytoskeleton proteins (NFTP). In that study, the authors strongly provided evidences for neural histogenesis of ES and suggested a close relationship between ES and peripheral neural tumors [59]. In the study from Noguera et al., the authors evaluated the capability of three extra-osseous ES cell lines to differentiate toward a neural and muscular direction upon stimulation with dibutylryl cyclin adenosine-monophosphate (db cAMP) and 5-azacytidine, respectively. A neural but not myoblastic differentiation was observed as all the cell lines expressed neural markers including chromogranin, S-100 protein, and glial fibrillary acidic protein [60]. More recently, it has been demonstrated that forced expression of EWS-FLI1 in a rhabdomyosarcoma cell line induced cell morphology changes resembling ES cell particularly through modulation of EWS-FLI1 target genes involved in neural crest differentiation [61]. In parallel, several studies explored the hypothesis of a mesenchymal origin of ES particularly through modulation of EWS-FLI1 expression in different cellular models. Studies conducted on mesenchymal stem cells (MSC) evidenced that forced expression of EWS-FLI1 blocked differentiation along osteogenic and adipogenic lineage, accordingly with the undifferentiated phenotype of ES, [62] as well as myogenic differentiation in a murine myoblast cell line [63]. Successive studies confirmed that marrow-derived mesenchymal cells could be the progenitor of ES as EWS-FLI1 expression induces acquisition of EWS-specific morphological features [64] and because these cells are particularly permissive for EWS-FLI1 oncogenic transformation also as unique event [64, 65]. On the opposite site, silencing of EWS-FLI1 in different ES cell lines caused convergence toward the MSCs gene profile and induced expression of specific MSC markers such as CD44, CD54, CD59, CD73. Moreover, EWS-FLI1 silencing induces ES cells to differentiate along the adipogenic or adipogenic lineage upon treatment with appropriate differentiation agents [66].
2.6 Molecular biology of Ewing sarcoma

Conventionally, bone sarcomas or soft tissues can be cytogenetically distinguished in two groups: one group characterized by simple near-diploid karyotype with few chromosome rearrangements and one group with a complex karyotype and a severe disturbance in genomic stability [67]. ES belongs to the simple karyotype sarcomas group as it carries a tumor-specific recurrent chromosome aberration leading to the fusion of the EWSR1 gene with one of several members of the ETS family of transcription factor [68], as previously cited. In 85% of the cases, patients express the t(11;22)(q24;q12) translocation which product is EWS-FLI1 chimeric protein [69]. In 10-15% of the cases, the translocation t(21;12)(22;12) leads to the fusion of EWSR1 to ERG while others translocations are rare and account 1 to 5% of the cases (Table 1).

**Table 1.** Translocations and fusion gene in ESFT.

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Fusion gene</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(11;22)(q24;q12)</td>
<td>EWS-FLI1</td>
<td>85</td>
</tr>
<tr>
<td>t(21;22)(q22;q12)</td>
<td>EWS-ERG</td>
<td>10</td>
</tr>
<tr>
<td>t(7;22)(p22;q12)</td>
<td>EWS-ETV1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>t(17;22)(q21;q12)</td>
<td>EWS-ETV4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>t(2;22)(q33;q12)</td>
<td>EWS-FEV</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

The chimera holds the amino terminus of EWSR1 gene and the carboxy terminus of the ETS gene acting as an aberrant transcription factor. EWSR1 gene, located on chromosome 22, belongs to a subgroup of RNA-binding proteins called the TET family and is ubiquitously expressed. Proteins of this family hold a central RNA-binding motif and three regions rich in glycine, arginine and proline, interacting with RNA [69]. EWS is a nuclear protein that appears to be recruited to promoter regions acting as a promoter-specific transactivator upon association with other factors [49] such as RNA
polymerase II, TFIID and CBP/p300 [70]. In addition, the N-terminus domain is rich in glutamine and it stimulates transcription if fused with DNA-binding domains, as described in ES [71]. In most of the cases, the fusion gene interests EWSR1 and FLI1, located on chromosome 11 (Figure 2). In particular, genes breakpoints most commonly interest exon 7 of EWSR1 and exon 6 of FLI1 (type 1 fusion), showing a lower transactivation potential than a second variant interesting EWSR1 exon 7 and FLI1 exon 5 (type 1 fusion). This lower transactivation potential appears to correlate with a higher relapse-free survival of ES patients [72]. The resulting fusion protein holds 264 amino acids of EWSR1 and 233 amino acids of the C-terminus portion of FLI1.

Breakpoints can occur in different sites of the genes and result in several isoforms of the chimera: variations interest 264-348 amino acids of EWS and 191-324 amino acids of FLI1. EWS-ETS fusion proteins act as aberrant transcription factors modulating the expression of target genes in a sequence-specific manner that is determined by the ETS component under the control of potent EWS transactivation component [49]. In vitro studies demonstrated that forced EWS-FLI1 expression is transforming in NIH3T3 murine fibroblasts while its silencing, via antisense RNA or short interfering RNA (siRNA), caused growth inhibition, apoptosis, decreased anchorage-independent growth and tumorigenicity in vivo [74-79]. By contrast, EWS-FLI1 expression in mouse embryonic fibroblasts or human primary fibroblasts failed to induce transformation and resulted in growth arrest and apoptosis [80, 81]. These last results underlie the importance of a specific cellular context for EWS-FLI1-mediated oncogenesis [73]. Beside not so extensively studied, all the EWS-ETS fusion proteins act as aberrant
transcription factors and induce the same transformed phenotype. Beside other EWS-ETS rearrangements have not been so extensively studied, evidences suggest that all EWS-ETS proteins act as aberrant transcription factors that can regulate gene transcription and transform cells [72]. Explanation of this common behavior could be the loose specificity of ETS proteins for target genes [82] and the contemporary high homology in the DNA-binding domain [72]. Nevertheless, some functional differences have been observed between the various translocations: oncogenic transformation in NIH3T3 cells could be induced by EWS-FLI1, EWS-ERG and EWS-FEV but not by EWS-ETV1 and EWS-ETV4 [83]. Another difference regards the location of the tumor as in one study 11 cases on 12 harboring EWS-FEV, EWS-ETV1 or EWS-ETV4 presented extraosseous tumors but explanations are unknown. Overall, further studies are needed to better understand these differences. In addition, non-EWS fusions have been identified in less than 1% of cases. TET proteins family includes EWS, TLS and TAF15 and TLS/ERG or TLS/FEV have been described in some ES patients adding complexity to the ES biology. It is generally assumed that TET-ETS proteins function in a similar fashion compared to EWS-FLI1 but further studies will be necessary [70]. As oncogenic processes are due to the aberrant transcriptional activity of the chimera, several studies have been performed to identify EWS-ETS target genes. From this point of view, RNA-interference (RNAi) based approaches, microarray technology and ChIP analyses have been useful tool to identify a large number of genes dysregulated by EWS-FLI1. Studies have demonstrated that NR0B1, NKX2.2, GLI1 are up-regulated by EWS-FLI1 and possess a critical relevance in oncogenic transformation. On the other side, EWS-FLI1 deregulates genes involved in cell proliferation, evasion of apoptosis, drug-resistance, cell cycle control, angiogenesis. Particularly, the chimera up-regulates PDGFC, IGF-1, MYC, CCND-1 e NKX2.2 while down-regulates p21, p57, TGFβRII and IGFBP3 [70]. Some studies suggest that EWS-FLI1 oncogenic activity may also be mediated by some DNA-binding-independent mechanisms. Indeed, expression of EWS-FLI1 molecules with point mutations or large deletions in the ETS in NIH3T3 cells were still able to induce tumors in mice [84].

Additional chromosomal changes that are frequently found in ES include gain of chromosome 1q (32%) and chromosome 2 (29%), trisomy of chromosomes 8 (67%), and 12 (29%), losses of 9p (23%) and 16q (32%) [85, 86]. In addition, patients with primary tumor with low copy number changes (≤ 3 copy number aberrations) show a significant better prognosis in terms of overall and event-free survival respect to those with a high number of alterations [86]. Homozygous deletions of CDKN2A, encoding p16INK4a, has been observed in 10 to 30% of the cases and is associated with poor prognosis when combined with co-presence of mutations on p53 [87]. Inactivating point
mutations of *TP53* are present in 4-14% of cases and are associated with poor outcome when compared to patients with wild type p53, defining another subgroup of patients with poor clinical outcome [88]. Recently, as performed in other tumor types, the genomic landscape of ES has been explored with next generation sequencing techniques in order to identify additional molecular alterations that could be associated with tumor aggressiveness. The results demonstrate that ES genome is very stable with a low somatic mutational rate, compared to other tumors. Indeed, results from three different groups highlighted just one genetic mutation more together with those previously described. In particular, they discovered inactivating mutations on cohesin complex subunit *STAG2* in 21.5% of tumors [89-91] and *STAG2* mutations were found associated with poor overall survival [91]. This low mutational rate could be due to the short amount of time of pediatric cancers to accumulate passenger mutations or to a preponderant epigenetic- more than genetic-driven oncogenesis in pediatric tumors. In addition, this could be a specific feature of fusion-driven cancers [89]. From the clinical point of view, this paucity of mutations represents a limitation for the identification of targetable pathways [90]. Overall, the results further highlight the importance of *EWS-ETS* rearrangements in this tumor type. As previously mentioned, EWS-FLI1 requires a specific cellular environment to induce oncogenic transformation. Some critical factors that have been identified include the presence of an intact insulin-like growth factors (IGF) system and the expression of CD99, a 32kDa integral membrane glycoprotein expressed in 90% of the cases. EWS-FLI1 directly affects at transcriptional level the expression of important components of the IGF system thus creating the loop IGF-1/IGF-1R sustaining cell growth [92]. CD99 acts as an oncogene in ES cells as triggering of this molecules induces apoptosis and inhibition of growth *in vitro* and *in vivo* and prevents normal neural differentiation [93, 94]. Considering its high expression, it is considered a diagnostic biomarker together with neuron-specific enolase (NSE), S-100, synaptophysin and, depending on the level of neural differentiation, vimentin, cytokeratin and neurofilament [73].

2.7 Treatment

ES treatment is based on a combination of chemotherapy, surgery, and radiotherapy [95]. The standard approach is given by local treatment of the tumor (surgery and/or radiotherapy) and cycles of systemic chemotherapy (pre- and postoperatively). Surgery
plays an essential role in treatment of primary tumor, especially to avoid radiotherapy, but it is not always feasible. Conventionally, in case of localized disease neo-adjuvant chemotherapy is applied for a local control and to facilitate surgery. Chemotherapy response is than monitored to evaluate clinical response and modulate adjuvant chemotherapy. Chemotherapy is used as a combination of six drugs: cyclophosphamide, ifosfamide, adriamycin, vincristine, dactinomycin D, and etoposide. Multicentric clinical studies demonstrated an higher efficacy for the association of 4 drugs including cyclophosphamide, adriamycin, vincristine, and dactinomycin D more than an association of 3 drugs or the single drugs alone. Patients with metastatic disease remain a therapeutic challenge. Patients with metastasis at diagnosis show a worse prognosis when treated with the same regimen utilized for localized disease. For these patients a more aggressive treatment is necessary and consists of higher doses and a reduced time between cycles of treatment followed by myeloablative therapy and stem-cells transplantation [56]. Overall, such aggressive treatment causes severe side effects. Therapy amelioration represents an urgent need for ES and particularly for patients with metastatic disease. Unfortunately, few new drugs are available for ES treatment and always are less effective than conventional drugs. Innovative therapeutics have been developed based on biological features of ES cells. In ES, IGF-1R represents an attractive target of both monoclonal antibodies (MAb), including AVE1642, CP-751,871, IMC-A12, and tyrosine kinase (TKI) inhibitors, including AEW-541 and ADW742. These agents showed good preclinical results but poor clinical effects with just a 10% of patients experiencing relevant benefits [96] especially for mechanisms of innate or acquired resistance. Considering its elevated expression in ES cells and its key role in ES malignancy, CD99 molecule has been considered as potential therapeutic target. Recently, a new anti-CD99 MAb scFvC7 has been described showing benefits based on its specificity to ES cells. Trabectedin, ET-743, is an alkylator agent with antitumoral activity in different tumors, particularly those bearing translocations. In myxoid liposarcoma, Trabectedin down-regulates the binding of FUS-CHOP fusion gene product to promoter regions of its target genes. Some studies demonstrated that, similarly, Trabectedin interferes with EWS-FLI1 activity [97]. In ES, clinical trials have been performed showing an insufficient activity of this agent as monotherapy but a good tolerability with low side effects [98, 99].
3. Prostate cancer

3.1 Epidemiology and risk

The first case of prostate cancer (PCa) was diagnosed by histological examination in 1853 by J. Adams, a surgeon at The London Hospital, who described this condition as “a very rare disease” [100]. Today, PCa is the second most diagnosed cancer in adult men and the sixth cause of death in males accounting for 14% of new cancer cases and 6% of cancer death worldwide. Incidence of PCa is 2 to 5 times higher in developed countries compared with developing countries as a result of a set of risk factors and diagnostic procedures [101]. Several risk factors have been identified during the years and, despite no preventable factors exist, a general knowledge about them should be maintained. Advanced age is the principle cause of PCa as most of the cases are men over the age of 65 while it is rarely seen in men younger than 40 years. Autopsy data indicate a 90% of prevalence in men ages 70 to 90 indicating more men will die with PCa more that for it [102]. The second most common risk factor is the race with the highest risk in Africans, intermediate in Caucasians and lowest among Asians [103]. The role of ethnicity is still unclear but may be related with a combination of socioeconomic, environmental and dietary factors. Family history of PCa is a third well established risk factor for the disease [104]. Particularly, the risk of PCa is two times increased in men with a first degree relative and is even higher if the relative was diagnosed at an age younger than 60 [105].

3.2 Prostate anatomy and histology

In men, prostate is a partly glandular and muscular organ surrounding the urethra at the base of the bladder. Together with seminal vesicles and bulbourethral glands, prostate represents an accessory gland of reproduction as its primary function is to secrete an alkaline fluid forming part of the ejaculate, which aids in motility and nourishment of the sperm. This fluid (pH 7.29) is rich in lipids, proteolytic enzymes, acid phosphatase, fibrolysin and citric acids. The smooth muscle part of prostate helps semen expulsion during ejaculation. The prostate gland is surrounded by the prostatic capsule and
neurovascular bundles outside of the capsule are responsible for erectile function. According to the classic work of McNeal [106-108], the prostate gland is characterized by three major glandular regions: central, transition and peripheral zones (Figure 3). A fourth zone is an anterior fibro-muscular zone lacking glandular components as it is composed of muscle ad fibrous tissue. The central zone refers a vertical wedge of glandular tissue lateral to each ejaculatory duct. A narrow band of stroma separates the central zone from peripheral zone. Transition zone was found to lie in the convexity of the peripheral zone. Differences between the three areas were described in terms of stroma and glandular architecture. At histological level, prostate contains a pseudostratified epithelium with three differentiated epithelial cell types: luminal, basal and neuroendocrine [109-111]. Luminal cells form a continuous layer of polarized columnar cells that produce protein secretions and express markers including cytokeratins 8 and 18 and high levels of androgen receptor (AR). Basal cells are located under the luminal cells and express p63, cytokeratins 5 and 14 but low or undetectable levels of AR. Neuroendocrine cells are rare cells, which function is unknown, expressing endocrine markers but no AR. In 75% of cases, PCa develops in the peripheral zone while benign prostatic hyperplasia (BPH) develops in the transition zone [112].

![Figure 3. Sagittal diagram of adult human prostate showing distal urethra (UD), proximal urethra (UP) and ejaculatory duct (E). Three major glandular zones are shown: central zone (CZ), peripheral zone (PZ) and transition zone (TZ).](image)
3.3 Diagnosis and staging

From a clinical point of view, patients with PCa at early stages are mostly asymptomatic. Lower urinary tract symptoms including weak stream, urgency, frequency, nocturia, incomplete emptying and incontinence may be present but are common with benign prostatic hyperplasia. Patients with PCa may also present hematuria, hematospermia and erectile dysfunction. Urinary symptoms must be accompanied by further analysis in order to distinguish PCa from inflammatory disorders or hyperplasia. In advanced disease, patients may present bone pain in different locations including hips, back and pelvis, or unexplained anemia [114]. In 95%, PCa refers to an adenocarcinoma, originating from prostate gland epithelial cells in peripheral zone and with luminal phenotype. However, other categories of PCa exist such as ductal adenocarcinoma, mucinous adenocarcinoma and neuroendocrine prostate cancer but are extremely rare [115]. Diagnosis of PCa is performed by a histologic evaluation of prostate tissue sampled from a prostate needle biopsy. However, the decision to perform a biopsy depends on prior Prostate Specific Antigen (PSA) evaluation and digital rectal examination (DRE) findings as well as on age, ethnicity and co-morbidities. PSA is a serine protease of the Kallikrein family produced by the prostate and a component of seminal fluid important for ejaculation. Serum evaluation of PSA for early PCa detection is part of the clinical practice since the 1980s [116]. Normal PSA value ranges from 0 to 4 ng/ml while it is found increased in case of PCa as well as BPH, infections, ejaculation within 48 hours of serum evaluation, trauma and age. For this reason, PSA serum evaluation remains an imperfect test. Nevertheless, prostate biopsy is recommended for men with a serum PSA ≥ 4.0 ng/ml regardless of DRE [117]. DRE is limited because it allows palpation of the posterior surface of the gland but it is performed regardless of PSA analysis results. Men with positive DRE are directed toward a biopsy independently on PSA serum levels. Considering the limitations of both PSA evaluation and DRE, research of new biomarkers for early diagnosis of PCa with characteristics of high specificity and sensitivity represent an urgent need for this tumor. Transrectal ultrasound-guided needle biopsy represents the main method to obtain prostatic tissue. The number of biopsies rage from 8 to 16 and most of the tissue is sampled from the peripheral zone. The most common complications of this procedure are hemorrhagic including hematuria, hematospermia and hematochezia [118] thus patients taking antiplatelet drugs should discontinue the treatment. Prostate biopsy is then categorized by the pathologists using a grading system known as the Gleason Scoring system. This system was described in the 1960s and it
characterizes prostate tumor architecture and morphology. In addition, Gleason score represents the strongest clinical predictor of PCa progression [119]. The Gleason system assigns a grade to the two largest areas in each biopsy specimen. Grades span from 1 to 5 where 1 is the least aggressive and 5 is the most aggressive. The two numbers are added and men diagnosed with a Gleason grade 7 or more are at increased risk of extraprostatic extension, recurrence after initial therapy and more likely to die for the disease. On the opposite side, men with Gleason minor that 7 have a low risk of cancer-specific death. In a study published in 2011 from Eggener et al. the overall 15-year PCa specific mortality rate was evaluated in a cohort of more than 10000 patients treated with radical prostatectomy. The results showed that the probability of death from PCa varied by the age of the patients ranging from 0.6-1.2% for Gleason 6 or less, 4.7-6.5% for Gleason 3+4, 6.6-11% for Gleason 4+3, and 22-37% for Gleason 8 or higher [120].

The staging of PCa is determined by PSA value, DRE findings, prostate biopsy results, and Gleason score and it is important to establish the treatment options for the patients. Staging is divided between clinical and pathological: clinical staging is based on clinical findings such as PSA, DRE and imaging while pathologic staging is based on tissue diagnosis and relies on the TNM system developed by the American Joint Committee on Cancer. The TNM system describes the extent of tumor (T), lymph node involvement (N), and presence of metastatic disease (M). Each category is further divided in subcategories. Extent of the tumor is divided into T1-T4 with higher T indicating higher involvement of the prostate and surrounding structures. Node category is divided in 0 or 1 indicating lymph node involvement. Metastatic disease is categorized in 0 if the disease is not spread or 1. Considering all these parameters, patients are classified as “low-“, “intermediate-“ or “high-“ risk.

### 3.4 History of prostate cancer

Heterogeneity and multifocality represent two main characteristics of PCa posing significant difficulties both at clinical and research level. Histological inspection of PCa tissue reveals the high heterogeneity of this tumor type as it is characterized by juxtaposition of benign glands, preneoplastic foci, and neoplastic foci of varying severity [115]. Regarding multifocality, it has been widely described that, within a section of PCa, individual genetically distinct neoplastic lesions, even in close proximity, can be present. This evidence suggests multiple neoplastic foci may emerge
and evolve independently [121]. The main mediator of PCa development, as well as normal prostate, is the AR. Particularly, AR sustains the proliferation of tumor cells with its transcriptional activity upon stimulation of androgens. The most abundant androgen is testosterone that is activated by 5a-reductase in dihydrotestosterone. In PCa, AR suppresses proliferation of basal cells, supports survival of luminal cells and promotes metastasis as demonstrated by studies in mice [122].

### 3.4.1 Cell of origin

Several studies have been conducted in mice to determine the cell of origin of PCa. Considering the luminal phenotype of human PCa, the cell of origin should correspond to either a luminal cell [123, 124] or a basal cell that can differentiate into luminal progeny following oncogenic transformation [125]. Particularly, studies of PSA-Cre; Pten\(^{\text{floxed}}\) mice suggested a luminal population corresponding to the cell of origin in this model [126]. Analysis of Probasin-Myc and Nkx3.1-Myc transgenic mouse lines suggested the same luminal origin [127]. In addition, histopathological evidence of MYC expression in preneoplastic lesion is in line with the luminal origin of this tumor as MYC is exclusively expressed by luminal cells but not basal cells [128]; similar results have been reported with respect to telomere shortening [129] and the androgen related-TMPRSS2-ERG fusion gene as AR is expressed by luminal cells [130]. On the opposite side, different studies reported strong evidences regarding the basal origin of this tumor. In the paper from Goldstein et al. it is demonstrated that injection of the mixture of urogenital sinus mesenchyme (UGSM) with human prostate basal or luminal cells into immunodeficient NOD(-)SCID(-)IL-2Rg/- mice induced adenocarcinoma when using basal cells but not luminal [125]. Another study conducted in Pb-Cre4; Pten\(^{\text{floxed}}\) mice showed an expansion of basal cells as well as intermediate cells coexpressing basal and luminal markers in tumors [131]. More recently, an analysis of basal and luminal epithelial populations from mouse prostate has shown that basal cells are more readily transformed by lentiviral expression of ERG and AR in tissue reconstitution experiments [132]. Overall, these data indicate that despite all the information obtained until now, further studies are necessary to better elucidate the origin of this tumor. It remains unclear, whether different cells of origin are used in PCa initiation [123].
3.4.2 Prostatic intraepithelial neoplasia (PIN)

PIN is a specific type of lesion that is believed to represent the primary precursor of human PCa beside this relationship has not been demonstrated conclusively [133]. At histological level PIN is characterized by the appearance of luminal epithelial hyperplasia, reduction in basal cells, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia, and nuclear atypia and increased expression of cellular proliferation markers, in case of high-grade PIN [123] (Figure 4). PIN can be found as low-grade or high-grade forms with high-grade form thought to represent the precursor of early invasive carcinoma as demonstrated by different evidences. PIN lesions are found in peripheral zone [134] and the features of high-grade PIN lesions generally precedes those of carcinoma by at least 10 year, according with the idea of cancer progression [135]. In addition, PIN lesions are multifocal as demonstrated by allelic imbalance analysis and chromosomal abnormalities found in PIN are similar to those of early invasive carcinoma [136, 137]. Architectural and cytological features look like those of invasive carcinoma including disruption of basal layer [138]. Eventually, PIN lesions express markers of differentiation commonly altered in early invasive carcinoma including E-cadherin and vimentin [139, 140]. On the opposite side, exclusive PIN characteristics include intact basement membrane [138], no PSA expression [140], similarity between PIN histological characteristics and premalignant lesions of the breast [141]. In addition, PIN lesions show architectural and cytological characteristics that are not believed to be precursor features of prostate cancer [123].

![Figure 4. Histopathology of human PCa. Hematoxylin-eosin-stained section of human (A) benign normal tissue, (B) PIN, (C) well-differentiated adenocarcinoma, (D) poorly-differentiated adenocarcinoma [123].]
3.4.3 Latent and clinical cancer

Prostate carcinogenesis is a process of 20-30 years or more starting as a proliferative inflammatory atrophy (PIA), passing through PIN and in some cases leading in a carcinoma. Evidences suggest that one of the cause predisposing to cancer development could be a prostate inflammation due to infectious agents or ingestion of carcinogens. In addition, an inherited genetic background could also make an individual more susceptible to prostate tumorigenesis but implicated genes must be discovered yet [142]. Clinically, PCa can be divided in two main groups: prostate tumors able to spread that will end up being lethal with a clinical relevance, and others indicated as latent that are relatively indolent [143]. Autopsy studies have indeed demonstrated that almost 70% of men have a tumor in the prostate at the time of the death but with no clinical relevance. It has been estimated that 15-30% of males over the age of 50 and 80% of males over 80 years of age harbor microscopic, latent PCa [144]. In addition, it is fatal for only 3% of men. From this point of view, PSA evaluation doesn’t represent an useful biomarker as it is informative of an organ- but not a disease-specific affection and PSA does not distinguish which type of PCa a man may have i.e. a cancer that will never cause a problem, a clinically relevant tumor that will cause morbidity and mortality if left in place or an incurable, metastasizing form [142]. Nowadays, the main challenges in the field of PCa research include the discrimination of the two forms of the disease, latent or clinically relevant, and consequently the identification of which men can be cured with treatment and which not require treatment avoiding treatment-induced morbidities.

3.4.4 Metastasis

The primary site for PCa metastasis is invariably the bone, where characteristics osteoblastic lesions are formed [145]. Secondary sites are represented by lung, liver and pleura [146]. Metastatic disease accounts for more than 90% of deaths associated with PCa and almost 90% of patients dead for PCa die of metastatic bone disease [147]. Most of bone metastasis are classified as osteoblastic, based on the radiographic appearance of the lesions indicating a deregulation of bone resorption and formation processes. Bone metastasis occur when PCa single cells, with acquired characteristics of motility and invasiveness, detach from epithelial sheet, reach the circulation, adhere to bone
marrow endothelial cells and migrate to the bone [148]. In patient, bone metastatic lesions cause severe bone pain, skeletal fracture, hypercalcemia, and spinal-cord compression [149]. Mechanisms by which PCa cells form preferentially bone metastasis and particularly osteoblastic skeletal lesions are overall unclear but numerous studies have been conducted. Firstly, according to Paget's seed and soil theory [150], several bone paracrine factors contribute to the tropism of circulating PCa cells to the bone mediating the interaction between disseminated cells and resident bone cells [148]. From this point of view, this process involves a variety of adhesion molecules expressed by endothelial cells and PCa cells including CXCR4 [151], cadherin 11 [152], monocyte chemotactic protein-1 (MCP-1) and its receptor CCR2 [153]. Secondly, osteoblastic lesion is the result of the release of factors that stimulate osteoblast proliferation, differentiation and consequently uncontrolled bone formation by metastatic cancer cells [154]. This last process is also due to the osteo-mimicry capability of tumor cells. It is still to be clarify whether cancer cells already possess osteomimetic phenotype or it is acquired in the bone marrow. Anyhow, PCa cells are able to express factors involved in normal bone development and remodeling. Osteoblastic differentiation is a complex process regulated by several factors including bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF)-1 and -2, transforming growth factor-β1 (TGFβ1) and TGFβ2, fibroblast growth factor (FGF), the transcription factor RUNX2 [148]. Interestingly, the same pathways are implicated in the activation of resident bone and bone marrow cells induced by PCa cells [155-157].

### 3.5 Molecular biology of prostate cancer

PCa progression is accompanied by genetic alterations and molecular pathways holding a specific significance in each stage. Emergence of large-scale sequencing studies of cancer genomes has allowed the identification of the elevated molecular heterogeneity of tumors including PCa [158]. Extensive genomic analysis in PCa have been performed and both copy number variations and translocations have been identified as the most common genetic alterations. One information that is still lacking is whether there is a temporal sequence associated with this events or they are casually related [123]. Mutations of AR represent one well-established alteration in this disease and they are mostly related with castration-resistant stage. AR is amplified in one third of the cases while 10-30% of tumors present gain-of-functions mutations. Recently, alternative
splice isoforms encoding constitutive active AR variants have been identified in castration-resistant tumors. In addition, some studies identified the endogenous expression of androgen synthetic enzymes in tumor tissue as a mechanism to activate AR [123]. Around 85% of PIN lesions and adenocarcinomas display loss-of-heterozygosity (LOH) of chromosome 8p21.2 causing the deletion of a region containing the NNX3.1 homeobox gene [159]. NNX3.1 is an oncosuppressor gene with a relevant role in prostate epithelial cells differentiation [160]. Accordingly, NNX3.1 expression was found low expressed in series of PCa and metastasis or completely lost in advanced cancers [161]. Studies conducted in mice with the purpose to elucidate the role of NNX3.1 in cancer initiation evidenced that NNX3.1 inactivation causes a defective response to oxidative damage while its expression in PCa cell line protects against DNA damage [162, 163]. MYC oncogene has been found amplified in a subset of advanced PCa [164] while other studies found an up-regulation of MYC in PIN lesions and carcinomas in absence of gene amplification suggesting an altered regulation of MYC [128, 165]. MYC overexpression in mice induces PIN lesions, carcinoma and metastasis [166] and expression of MYC is sufficient to immortalize human prostate non-tumorigenic cells [123]. In 2005, the group of Tomlins et al. described chromosomal rearrangements involving the androgen-responsive promoter of TMPRSS2, on chromosome 21, and one of the ETS transcription factor family gene. The most common of these rearrangements involves ERG, located on chromosome 21, and results in the TMPRSS2-ERG fusion gene. This rearrangement, which can be due both to deletion or translocation, causes an androgen-related over expression of transcription factor ERG. A small percentage of cases contains fusions with other ETS transcription factors as well as with promoter fusion partners other than TMPRSS2. Fusion genes have been identified involving ETV1 and ETV4 ETS genes while TMPRSS2 was found replaced by untranslated regions from the prostate-specific androgen-induced gene SLC45A3 [167]. TMPRSS2-ERG is expressed in 15% of PIN lesions and in 50 to 70% of localized PCa cases indicating it is an early event in this tumor [29]. Some evidences indicate that the formation of this rearrangement could be due to the androgen receptor activity itself that induces chromosomal proximity between the two genes following DNA damage [130]. Alternative splicing events of the initial fusion transcripts induce formation of more than eight fusion types which more frequently include the designated type III form involving TMPRSS2 exon 1 fused to ERG exon 4 and the type VI interesting TMPRSS2 exon 2 fused with ERG exon 4 [35]. It has been demonstrated that cases expressing type VI isoform were more aggressive than those expressing type III [168]. Studies exploring the functional significance of truncated ERG protein are controversial but suggest that ETS activation promotes epithelial-mesenchymal
transition (EMT) and invasiveness [169-171]. Nevertheless, TMPRSS2-ERG has been reported as insufficient to induce a transformed phenotype but instead to cooperate with other mutations [172]. The product of the fusion gene in PCa is a constitutive expression of ERG with a consequent deregulation of transcriptional pattern of the cells expressing the fusion gene. ERG was found particularly up-regulated in peripheral zone compared to transitional zone and analysis of deregulated genes indicated that TMPRSS2-ERG-negative tissues were more similar to normal control, while TMPRSS2-ERG-positive tissues displayed distinct deregulation of transcription [173]. Several studies have been focused on identification of target genes trying to elucidate mechanisms mediating malignancy. ChIP analysis pointed out that PIM1 oncogene is a direct target of ERG and that the consequent overexpression of PIM1 modifies cyclin B1 levels in TMPRSS2-ERG-positive cells [174]. In vitro and in vivo studies evidenced that ERG binds osteopontin (OPN) promoter. OPN is an extracellular matrix glycoprotein involved in the metastasis which is up-regulated by ERG [175]. Other target of TMPRSS2-ERG are represented by cysteine-rich secretory protein 3 (CRISP3), CACNA1D, PLA1A [176] but studies in this field are ongoing.

PTEN (phosphatase and tensin homolog deleted on chromosome ten) is a tumor suppressor gene frequently altered in cancer and its protein represents a key mediator of the oncogenic phosphoinositide 3 kinase (PI3K) pathway. A copy number loss of PTEN has been described in PCa and it is considered as an early event in prostate carcinogenesis [177] while PTEN loss in mice results in PIN or adenocarcinoma [178]. Loss of PTEN has been described to cooperate with other mutations including loss of NKP3.1, up-regulation of c-Myc, and TMPRSS2-ERG fusion. More recently, recurrent mutations with a role in PCa aggressiveness were found in SPOP, FOXA1 and MED12 genes [179, 180]. Up-regulation of Akt/PI3K pathway has been described in PCa as a consequence of PTEN loss but also because of mutations interesting Akt1 [181] or the p110β isoform PI3K [182]. Deregulation of this pathway has been particularly associated with castration-resistant disease. In parallel, the MAPK signaling pathway, including ERK (p42/44) and RAS or RAF, has been frequently found activated in advanced disease [123]. Other alterations frequently found in PCa include deregulation of oncogenic tyrosine kinases like Her2 or SRC [183, 184], up-regulation of the EZH2 gene, encoding for a histone lysine methyltransferase, which amplification has been associated with aggressive tumors [185, 186]. In addition, the role of microRNAs (miRNAs) in PCa has been widely investigated. In studies of miRNA expression profile, a pattern discriminating between indolent from aggressive disease was evidenced [187] as well as specific miRNAs were associated with castration-resistant PCa [188]. miRNAs have been evidenced as regulators of important gene in PCa such
as PTEN, which expression is negatively regulated by the cluster miR-106b-25, or EZH2 that is regulated by miR-101 [189]. In addition several miRNAs have been associated with androgen receptor signaling including miR-125b, miR-21, miR-141, others were found to be involved with cancer-related cell migration such as miR-141, miR-143, miR-145. In addition, a serie of miRNAs was found associated with metastasis: down-regulation of miR-16, miR-32a, miR-126*, miR-205, miR-146a was found associated with metastasis while an up-regulation was found for miR-301 and miR-125b [190]. Overall, data from molecular biology underlie the heterogeneity of this tumor type and evidence that PCa can be considered as a collection of cancers [191] characterized by sets of molecular alterations. The concept of molecular classification of PCa can be useful in the perspective of personalized medicine as well as of identification of subgroup of patients with different prognosis.

3.6 Prognosis and treatment

Potential prognostic factors have been investigated in PCa at clinical, pathological and molecular level. Identification of prognostic biomarkers represents one of the main challenges in PCa management especially to discriminate between indolent tumors, which can be controlled by active surveillance, and tumors with aggressive behavior requiring more radical treatment strategies. Patients are divided in risk groups predicting biochemical relapse free survival, indicating increase in serum PSA after treatment, or clinical relapse free survival [192]. The strongest clinical predictor of PCa is the Gleason score where patients with Gleason 7 or more show increased risk of recurrence or death after initial therapy. PSA levels at diagnosis is a standard risk factor for patients stratification. Increased PSA at diagnosis is associated with poor outcome after treatment [193]. Clinical, pathological and lymphnode pathological stages and margins positivity are also considered determinants of PCa poor prognosis [194]. Together with clinical parameters, many molecular and genetic factors have been investigated to better individualize risk prediction [193] in cohort of patients undergone radical prostatectomy or radiation therapy. First, numerous studies evaluated the association of TMPRSS2-ERG and outcome of PCa patients obtaining controversial results. Recently, the study conducted by Hägglöf et al pointed out an increased risk for PCa specific death for patients expressing the fusion gene [195]. This data is not in accordance with previous results obtained in two different studies [194, 196]. Nevertheless, the study by Rubio-Bronics et al. pointed out that, beside without affecting prognosis, TMPRSS2-ERG status classifies patients into groups defined by different clinico-pathological prognostic
factors with PSA, Gleason and margin status as prognostic factors in patients expressing the fusion gene while clinical stage (cT), Gleason and margins displayed prognostic relevance in non-expressors [194]. Molecular markers of PCa prognosis include Ki67 [197], loss of PTEN [198] or Akt mutations [199]. More recently, high speckle-type POZ protein (SPOP) gene expression was found statistically associated with favorable biochemical and clinical progression free survival [179] while elevated insulin-like growth factor 2 binding protein 3 (IGF2BP3) serum levels were found associated with poor prognosis [200]. In addition, miRNAs prognostic value was considered as well by several studies. Particularly, a recent study evidenced miR-187 and miR-182 as promising prognosis biomarkers in PCa [201]. Prognosis makers expression, extent of the disease and patient age support the clinician in the decision of the most appropriate treatment. Despite guidelines exist, the main purpose of all the efforts in the field of biomarkers research is to address the patient toward a personalized treatment. Consequently, patients will receive the most effective treatment with less side effect avoiding useless and expensive therapies. Therapeutic options are thus different considering, first of all, whether patient is affected by localized or advanced disease. In case of localized disease, the therapeutic options are radical prostatectomy, radiotherapy or cryotherapy. Surgical intervention for definitive treatment of PCa includes open radical prostatectomy, laparoscopic radical prostatectomy and robot-assisted laparoscopic radical prostatectomy. During the intervention the entire prostate gland and seminal vesicles are removed. Radical prostatectomy side effects are erectile dysfunction (ED) and urinary incontinence (UI). External beam radiation therapy (XRT) means to deliver a curative dose of radiation to the prostate without damaging surrounding tissues including bladder, rectum and bowel. Radiation therapy is a good option for patients who are poor surgical candidates and radiation regimens must be formulated based on risk levels. Side effects include urinary urgency and frequency, dysuria, diarrhea, proctitis. Brachytherapy involves placing radioactive sources into the prostatic tissues via needles, seeds, or wires under transrectal ultrasound guidance. Rates of brachytherapy can be high- or low-dose where the low-dose-rate consists of permanent implantation of “seeds”, whereas high-dose-rate is temporary. This therapy is indicated for low-risk disease. Side effects are the same of XRT plus urinary retention. Cryotherapy is a surgical intervention involving freezing of the gland. A cryoprobe is inserted into the prostate through ultrasound guidance to a temperature of -100° to -200° for 10 minutes. Localized high-risk disease where prostatectomy is not possible is indicated for this treatment. Complications include ED, UI, urinary retention, rectal pain, fistula. Advanced disease refers to recurrent PCa following therapy, locally recurrent disease, systemic recurrence, or clinical recurrence. Hormone therapy goal in
PCa is to reduce the levels of male hormones preventing the activation of AR and is indicated for patients with advanced non metastatic disease. Castration can be carried out surgically with orchiectomy or chemically with luteinizing hormone-releasing hormone (LHRH) agonists. LHRH agonists most commonly used are leuprolide, goserelin, triptorelin and histrelin and they can be used alone or in combination with nonsteroidal antiandrogen like flutamide, bicalutamide, nilutamide. Unfortunately, response to chemical castration lasts for 2 or 3 years before PSA begins to rise and in that case patients are considered to have hormone refractory disease. In case of refractory disease, secondary hormone therapy represents a therapeutic option. Overall, hormone therapy has a significant impact on quality life with both acute- and long-term effects. Acute side effects include fatigue, hot flashes, flare effects while long-terms effects include cardiovascular disease, anemia, osteoporosis, sexual dysfunction. Chemotherapy is used for the treatment of hormone refractory metastatic PCa and the standard of care is docetaxel-based regimen. Docetaxel is administered in combination with prednisone or mitoxantrone. Adverse side effects are myelosuppression, hypersensitivity reaction, gastrointestinal upset, peripheral neuropathy. Patients with bone metastasis should be considered for biphosphonate therapy with zoledronic acid. Emerging therapies for the treatment of hormone refractory metastatic PCa are cabazitaxel, abiraterone acetate, denosumab. Cabazitaxel is a microtubule inhibitor approved for patients who have already been treated with docetaxel. Adverse effects include neutropenia, gastrointestinal disturbance, renal insufficiency. Abiraterone acetate is a second-generation anti-androgen drug that blocks the synthesis of androgens through the inhibition of 17 α-hydroxylase/C17,20 lyase (CYP17A1). Denosumab is a monoclonal antibody targeting RANKL, a protein involved in bone destruction. Side effects indeed are hypocalcemia and osteonecrosis of the jaw [202].
4. The IGF system

The insulin-like growth factor (IGF) system includes three ligands (IGF-1, IGF-2 and insulin), three receptors (IGF-1R, IGF-2R and insulin receptor - IR), and 6 IGF binding proteins forming a family of extracellular proteins that bind IGF-1 and IGF-2 thus regulating their bioavailability and activity [203, 204] (Figure 5). The IGF system plays a pivotal role in normal growth and development and it mediates several aspects of malignant phenotype in a variety of human malignancies [205].

![Figure 5. Schematic representation of IGF system [206].](image)

4.1 The receptors

The IGF system includes IGF-1R, IGF-2R, IR and hybrid receptors. IGF-1R and IR evolved from an ancestral gene and display an elevated homology rate: 45 to 65% in the ligand binding domains and 60 to 85% in the tyrosine kinase and substrate recruitment domains. Together the two receptors control metabolism, growth, differentiation and nutrient availability [207]. Beside the elevated homology, IGF-1R and IR carry out different functions inside the cell. Particularly, IGF-1R regulates cell proliferation while IR has a metabolic role. *In vivo* studies demonstrated that null mutants for *IGF-1R*
exhibit a severe growth deficiency (45% normal size) and die early for organ hypoplasia [208] while mice lacking IR display an almost normal size (80-90%) but die for severe hyperglycaemia and hyperketonaemia consistent with the preponderant metabolic actions of IR [209]. The IGF-2R or cation-dependent mannose-6-phosphate receptor does not share a phylogeny with IGF-1R and IR. It is considered an oncosuppressor as down-regulates IGF-2 modulating its availability. In addition, loss of IGF-2R induces an increase of body size in mice [210, 211].

4.1.1 IGF-1R

IGF-1R is a transmembrane tyrosine kinase receptor (RTK) constitutively expressed in most cells and tissues and which role is mediate long term actions on growth and development [212]. IGF1R gene, mapping to chromosome 15q25-26, comprises 21 exons and spans more than 100 kb. Control of IGF-1R gene expression takes place both at transcriptional and post-transcriptional levels. Cloning studies pointed out that IGF-1R promoter is regulated by cis- and trans-acting factors under physiological and pathological conditions. The promoter is highly GC-rich (around 80%) and lacks TATA and CCAAT boxes, normally required for efficient transcription initiation. Despite absence of these motifs, transcription of IGF-1R is initiated within a unique initiator element located around 1000 bp upstream of the ATG translation start codon [213]. The gene codifies for a single 180 kDa chain and IGF-1R cDNA is composed of 4101 nucleotides and predicts a 1367 amino acid precursor, including a 30 amino acid signal peptide which is removed during translocation of the polypeptide chain. The 1337 amino acids left undergo a cleavage of the Arg-Lys-Arg-Arg sequence at position 707. The resulting propeptide is glycosylated, dimerized and translocated to the Golgi apparatus where it is cut by furin enzyme in α and β subunits [214]. Two αβ precursors are attached by disulfide bonds forming heterotetrameric IGF-1R complex (β-α-α-β) that is transported to the cytoplasmic membrane [215]. The N-terminal portion of IGF-1R is glycosylated [216]. The mature IGF-1R is constituted by two α chains spanning 130-135 kDa and two β chains spanning 90-95 kDa, attached by disulfide bonds α-β, α-α [217]. The two α subunits are completely extracellular and contain the ligand binding sites. IGF-1R binds IGF-1 and IGF-2, but not insulin, with high affinity. Studies based on the surface plasmon resonance technology demonstrated a difference of 4-fold in the IGF-1R affinity for IGF-1 compared to IGF-2 [218]. The two β subunits are composed
of an extracellular portion containing 5 glycosylated sites, a transmembrane hydrophobic region and the intracellular region holding domains involved in signal transduction: juxtamembrane domain, tyrosine-kinase domain (TK), ATP binding site and C-terminal portion [219].

4.1.2 IR

IR is expressed in liver, adipose tissue, skeletal muscle but also in brain, heart, kidney, pulmonary alveoli, pancreatic acini, placenta vascular endothelium, monocytes, granulocytes, erythrocytes, and fibroblasts [207]. This wide expression suggests that it is functionally involved in multiple mechanisms, in addition to the metabolic role. IR gene is located on chromosome 9 and it spans 120 kb containing 22 exons. The cDNA was isolated by the groups of Ullich and Rutter in 1985 and revealed two insulin receptor proreceptors sizes of 1343 and 1355 amino acids, respectively. This difference is due to the inclusion or not of a 12-amino acids segment at the C-terminal end of the IR α subunit. This segment is codified by exon 11, spanning 36 bp, that could be alternatively spliced [220]. As a consequence, IR exists as two isoforms: isoform A (IR-A), lacking exon 11, and isoform B (IR-B) containing exon 11. As IGF-1R, IR is a heterotetrameric protein composed of two extracellular α subunits and two transmembrane β subunits attached by disulfide bonds. The α and β chains are synthesized by the same mRNA encoding for 1370 amino acids. The protein is cleaved by furin into α and β subunits, glycosylated and processed into the Golgi apparatus. α-subunit contains 723 amino acids, with a molecular mass of 130 kDa while β-subunit is composed of 620 amino acids with a molecular mass of 95 kDa. Similarly to IGF-1R, the two subunits are involved in ligand binding and signal transduction, respectively. The two isoforms of IR display different binding affinity. Insulin is equally bound by IR-A and IR-B (EC₅₀=0.2±0.2 nM e EC₅₀=0.3±0.4 nM, respectively) [221]. A substantial difference regards the binding of IGF-2 where IR-A displays a higher affinity for IGF-2 compared to IR-B (EC₅₀=0.9±0.4 nM e EC₅₀=11.0±5.0 nM, respectively) [221]. None of the two isoforms is able to bind IGF-1 (EC₅₀>30 nM) [222]. Exon 11 inclusion causes more differences between the two isoforms. IR-A is the predominant IR isoforms in fetal tissues where mediates mitogenic effects while IR-B appears in postnatal life in insulin-target tissues including liver, muscles, adipose tissue regulating glucose metabolism [223]. Recently, IR-A has been found up-regulated in different tumor types accordingly to studies performed on murine 32D cells indicating
that IR-A induces mitogenic and anti-apoptotic signals while IR-B induces differentiation stimuli [224]. Considering that IR-A is capable to bind both insulin and IGF-2, it has been demonstrated that the receptor induces mitogenic signals upon IGF-2 binding while metabolic upon insulin binding [207].

4.1.3 Hybrid receptors

In cells expressing both IGF-1R and IR, hybrid receptors (HRs) can be found as α-β IGF-1R subunit can heterodimerize with one α-β of IR. In addition, IR isoforms can form heterodimers IR-A/IR-B. In this last case, it has been demonstrated that IR-A/IR-B hybrids recruit intracellular partners upon insulin or IGF-2 binding with the same affinity as IR-A homodimers while affinity for IGF-1 is low. The data suggest that, in presence of IR-A, IR-B hemireceptor is incorporated into hybrids with the consequence that most insulin binding sites behave as IGF-2 binding sites [207]. Heterodimerization occurring between IGF-1R and IR is mainly due to their high homology, spanning 27 to 84% depending on the region [225, 226]. The amount of hybrids is function of the mole fractions of each receptor but in some tumor tissues the proportion of HRs is higher than the expected suggesting there are still more unknown factors that can influence homo- and heterodimers formation. HRs can involve IGF-1R/IR-A (HR-A) or IGF-1R/IR-B (HR-B) presenting different biological characteristics. Studies demonstrate that both HR-A and HR-B bind IGF-1 with elevated affinity (EC₅₀ = 0.3±0.2 nM and EC₅₀ = 2.5±0.5 nM, respectively). Medium-high affinity occurs between insulin or IGF-2 and HR-A (EC₅₀ = 3.7±0.9 and EC₅₀ = 0.6±0.1, respectively) while they do not bind HR-B (EC₅₀ > 100 nM and EC₅₀ = 15.0±0.9 nM, respectively) [221]. In vitro studies exploring the HRs capabilities to phosphorylate a specific intracellular substrate, Crk-II, show that binding of the three ligands to HR-A determines Crk-II phosphorylation while IGF1 and IGF-2 only induce its phosphorylation upon HR-B binding. These results demonstrate that hybrids expression modifies insulin functional role as it induces mitogenic more than metabolic signals through HR-A activation. Modulation of insulin effects represent a main hypothesis regarding the physiological role of the HRs [207].
4.2 Ligands and binding proteins

IGF-1 and IGF-2 display a high homology with insulin structure and they control cellular and tissue growth [227]. IGF-1 is mainly produced by the liver under the control of the pituitary growth hormone (GH) but it can also be expressed by other organs with paracrine and autocrine effects [228]. For this reason, IGF-1 can be considered both a circulating hormone and a growth factor. IGF-1 gene is located on chromosome 12 and its expression is finely regulated in the liver. IGF-1 gene expression is induced by the GH, which production is regulated by the pituitary gland upon the regulation of the hypothalamic factors somatostatin and growth hormone-releasing hormone (GHRH). With a feedback mechanism, IGF-1 inhibits GH release acting on hypothalamus and pituitary gland. The protein is a single chain of 7.5 kDa containing 70 amino acids. IGF-1 expression increases from birth to puberty, when stimulates growth and differentiation of different tissues including bone [229] while decreases in the adult. Similarly to IGF-1, IGF-2 is expressed by liver and other tissues in adults but its expression is not under GH control [230]. IGF-2 gene expression is controlled by genetic imprinting and it is expressed by paternal chromosome under the control of the differentially methylated region (DMR) associated with the H19 gene, located upstream on chromosome 11. When DMR is methylated IGF-2 is expressed [231]. Recently, a family of proteins called insulin-like growth factor 2 mRNA binding proteins (IGF2BP1, 2, 3) has been found able to bind IGF-2 mRNA thus affecting its expression. Particularly, binding of IGF2BP3 to IGF2 mRNA induces a stronger expression of the ligand with stimulatory proliferative effects in different tumor types [232]. At protein level, the two ligands share high homology (62%) but IGF-2 is composed of 67 amino acids and it is expressed during life and its plasmatic levels are 3 to 7 folds higher that IGF-1. It induces proliferative and anti-apoptotic signals but it also plays a role in embryonic and fetal growth as well as in growth and differentiation of different tissues including muscle [233]. Insulin is a hormone produced by islets of Langerhans β cells in pancreas. Its active form is given by two chains: chain A, including 21 amino acids, and chain B, including 30 amino acids, cross linked by disulfide bonds. Insulin explicates mainly metabolic effects inducing glucose up-take of liver, adipose tissue and muscle upon hematic glucose increase. Specific amino acids confer to IGF-1 and IGF-2, but not insulin, the capability to bind a family of binding proteins (IGFBPs) which known components are 6. The IGFBPs hold IGF-dependent and –independent functions. In fact, they modulate half-life and bio-availability of IGFs: on one side they control the IGFs translocation toward specific tissues (IGFBP-1, -2, -4) but they also sequester the ligands preventing the binding to the receptor:
IGFBP-6 binds IGF-2 with high affinity while IGFBP-3 binds preferentially IGF-1. IGF-independent mechanisms include the inhibitory effect of IGFBP-3 on cell proliferation and migration regardless of IGF-1 binding [234].

4.3 IGF-1R: signal transduction pathways

IGF-1R activation induces proliferative and anti-apoptotic stimuli to the cell. For this reason, IGF-1R effects are crucial for the correct embryonic and postnatal development in humans but are deleterious in the context of cancer as IGF-1R with its activity has been found involved in proliferation, survival, migration, metastasis and chemoresistance mechanisms in different tumor types. Upon the binding of IGF-1 and IGF-2, the kinase domain of IGF-1R is activated inducing the auto-transphosphorilation of three tyrosine residues in the TK domain (Tyr 1131, 1135, 1136). Consequently, the activated TK domain phosphorilates several IGF-1R residues and different intracellular substrates involved in signal transduction. In particular, phosphorylated tyrosine residue 950 in the juxtamembrane domain serves as a docking site for IGF-1R substrates holding the Phosphotyrosine Binding Domain (PBT). The substrates include the family of insulin receptor substrates (IRS) 1-4 and Shc proteins [218]. These substrates initiate phosphorylation cascades that serve to transmit the IGF-1R signal.

![Image](image.png)

**Figure 6.** Representation of IGF-1R signal transduction [206].
These proteins are recognized by down-stream effectors holding the Src-homology-2 (SH2) domain such as PI-3K, Grb2/SOS, Ras GTPase activating protein [218]. The phosphatidylinositol 3-kinase is composed of a p85 regulatory subunit and a p110 catalitic subunit that, upon IRS-1 activation, phosphorilates phosphatidylinositol-(4,5)-biphosphate (PIP2) into phosphatidylinositol-(3,4,5)-trisphosphate (PIP3). PIP3 activates down-stream substrates including protein kinase B (Akt) by phosphorilations on threonine 308 and serine 473. Akt phosphorilation, in turn, regulates metabolic enzymes such as glycogen synthase kinase 3 (GSK3), modulates apoptotic regulators like Bad and caspase 9, and activates the mammalian target of rapamycin (mTOR) pathway thus affecting protein synthesis through the p70 S6 kinase and the Elongation Factor 4 binding protein-1 (4E-BP1).

In parallel, IGF-1R leads to activation of Ras pathway. Ras is a GTP protein that is activated when binds GTP while inactivated when binds GDP [235]. Ras is recruited and activated by Grb2/SOS (ras guanine nucleotide exchange factor) complex: Grb2 is a docking protein that can be activated both by IRS-1 or Shc. The active form of Ras leads to activation of the serin/threonine kinase Raf1 and consequently MAPK (Mitogen Activate Protein Kinase) family components including MEK1 and ERK1/2. Translocation of ERK1/2 to the nucleus results in activation of a transcriptional program leading to cellular proliferation.

In this way, IGF-1R controls different key points of cell cycle including the G0-G1, through p70S6 kinase activation determining ribosome synthesis and entrance into the cell cycle [236]; the G1-S progression is promoted by cyclin D1 increase and CDK4 expression that, in turn, phosphorilates pRb thus inducing E2F release and cyclin E synthesis [237, 238]. Particularly, cyclin D1 control represents the main IGF-1R mechanism of cell cycle progression control: cyclin D1 synthesis is indeed mediated by both ERK1/2 and PI3K/Akt pathways. ERK1/2 regulates its transcriptional expression while PI3K/Akt pathway stabilizes cyclin D1 mRNA thus favoring its protein synthesis. In addition, IGF-1R regulates G2/M transition by promoting synthesis of cyclins A and B, and cdc2 [218]. IGF-1R also avoids apoptosis induced by different agents including hypoxia, osmotic stress and anti-tumor drugs [239]. Particularly, PI3K/Akt pathway induces inhibitory phosphorylation of pro-apoptotic factors, including Bcl-2 family member Bad [240], and caspase 9 [205]. Moreover, active Akt phosphorilates FoxO transcription factor family members regulating cellular functions including cell growth inhibition, and apoptosis induction through the anti-apoptotic protein Bim expression. Upon Akt-induced activation, FoxO proteins translocate to the cytoplasm where undergo ubiquitin-induced degradation. IGF-1R thus suppresses the FoxO proteins transcriptional program. The MAPK pathway-induced apoptosis inhibition is given by
ERK1/2-mediated activation of several substrates including c-Myc, a Bcl-2 repressor, and STAT3, inducer of anti-apoptotic proteins like Bcl-xL, Mcl-1, survivin. Depending on the cell type, IGF-1R can also directly phosphorylate the Janus kinases (JAK)-1 and -2 that are involved in cytokine-mediated signaling [218, 241]. Phosphorylation of JAK proteins can lead to activation/phosphorylation of STAT proteins. In particular, STAT3 represents a very important mediator of IGF-1R transforming potential [242].

4.4 IGF system and cancer

The IGF system and, particularly, IGF-1R plays a critical role in various malignancies. Indeed, increased expression of IGF-1, IGF-2 and IGF-1R have been documented in different tumor types including glioblastomas, neuroblastomas, meningiomas, carcinomas of breast, gastrointestinal tract, ovarian, and Ewing sarcoma [218]. IGF-1R involvement in neoplastic transformation was first demonstrated by Sell et al. Particularly, the authors conducted their study on fibroblast-like cell lines from mouse embryos homozygous for a targeted null mutation of the IGF1R gene. These cells showed refractoriness to semian virus 40 (SV40) large tumor antigen (TAg)-induced transformation compared to control cell lines, highly sensitive to SV40 transforming effects [243]. Several following studies demonstrated the resistance of this cellular model to the transformation induced by other viral and cellular oncogenes: active Ras, E5 protein from bovine papilloma virus, E7 protein from human papilloma virus, EWS-ETS fusion protein in Ewing sarcoma, viral oncogene v-src, over-expression of receptors like EGF-R, IR, PDGF-Rβ and IRS-1. In addition, restoration of IGF-1R expression made the cells susceptible to the transformation [244]. Conclusion of these data is that IGF-1R is necessary for cell transformation but not sufficient as it must cooperate with other agents (chemical, biological or phisical). In addition, the role of IGF-1R in cancer is not through its over-expression but through its presence [245]. IGF-1R-mediated tumorigenesis can be due to genetic or functional alterations. Genetic alterations affecting IGF-1R include amplifications, and alterations of receptor regulators. IGF-1R gene amplification is a rare event but it has been described in melanoma [246], primitive breast cancer [247], pancreatic adenocarcinoma [248].

Mostly, altered IGF-1R expression is induced by mutations of its transcriptional regulators. Transcription factors associated with IGF-1R control include both
stimulatory and inhibitory factors that have been identified in cancer. Inhibitory transcription factors include p53, WT1 and BRCA1.
p53 is a nuclear transcription factor that is activated in response to DNA damage or hypoxia and it acts as an oncosuppressor as its activation leads to cell cycle blockade and apoptosis. p53 regulates gene expression of several components of the IGF system: it up-regulates IGFBP-3 [249] while down-regulates IGF-1R [250], IR [251], IGF-II [252] and IRS-3 [253]. p53 mutations, occurring in 50% of tumors, cause loss of IGFBP-3 and over-expression of IGF-1R, IGF-II, IR e IRS-3. In addition, loss of p53 up-regulates IGF-1R through MDM2 recruitment and decreased IGF-1R ubiquitination. In turn, IGF-1R can regulate MDM2 expression and increase wild-type p53 ubiquitination [254]. WT1 is an oncosoppressor that inhibits gene expression of IGF-1R and IGF-2.

In Wilm’s tumor and in breast cancer, WT1 mutations or deletions cause IGF-1R and IGF-2 over-expression [250, 255]. More recently, several studies have demonstrated that IGF-1R undergoes nuclear translocation and it directly interacts with DNA. Moreover, IGF-1R binds IGF-1R gene promoter thus establishing an IGF-1R autoregulation mechanism as demonstrated in breast cancer cells thus sustaining its own expression [213, 256]. Studies in breast cancer demonstrated that IGF-1R is able to transactivate the IGF-1R promoter in ER-negative cells. It is still unknown if IGF-1R autoregulation is a general mechanism of gene regulation [256].

BRCA1 is a nuclear phosphoprotein with transcriptional activity and it is involved in regulation of the G1-S and G2-M transitions acting as an oncosuppressor [257]. In breast cancer, wild type BRCA1 represses IGF-1R transcription while truncated form of BRCA1 is unable to decrease IGF-1R promoter activity [258]. Consistently, BRCA1 mutation carrier patients display higher IGF-1R levels than sporadic breast tumors [259]. Stimulatory transcription factors include EWS-WT1 chimera in desmoplastic small round cell tumor. This tumor is characterized by a chromosomal rearrangement involving EWS gene and WT1, on chromosome 11. The resulting chimeric protein possesses gain-of-function transcriptional activity and it was shown to bind WT1 cis-elements and to transactivate the IGF-1R promoter [260].

Functional alterations of IGF-1R refers alterations of IGFBPs, down-stream substrates, phosphateses regulating IGF-1R and its signaling, and IGF-2R.

Tumor progression can be due to a reduced expression of IGFBPs that modulate IGFs availability both in circle and in tumor microenvironment. Decrease of IGFBP2 causes an elevated mitogenic capability as IGF-1 and 2 are available for the receptor binding. It has been demonstrated that proteases or caspases from tumor cells can digest the IGFBPs allowing ligand release [261]. Between the six IGFBPs, IGFBP-3 is the most
commonly lost in tumor cell lines [218]. An exception is given by IGFBP-2, which increased expression is associated with malignancy in glioma and other tumors [262, 263].

IGF-1R activity is altered by increased expression or constitutive activation of downstream effectors [218]. IRS-1 has been found constitutively activated in breast cancer, leiomyosarcoma, Wilms tumor, rhabdomyosarcoma, liposarcoma [264] while IRS-2 is over-expressed in pancreatic tumor [265] or constitutively phosphorylated in metastatic breast cancer compared to non metastatic cells [266]. In liver tumor, an up-regulation of IGF-II, IRS-1 and IRS-2 has been reported [267] as well as constitutive activation of ERK1/2 has been reported to reduce tumor IGFs- and IGF-1R-dependence [268].

Phosphateses regulating IGF-1R frequently altered in tumor include PTEN. PTEN attenuates the signaling of different tyrosine kinase receptors including IGF-1R as it dephosphorilates PIP3 thus blocking Akt activation. Loss of PTEN induces a constitutive activation of PI3K pathway and this mechanism has been described in different tumors such as prostate, lung, stomach cancers [269, 270]. Protein tyrosine phosphatase 1B (PTP-1B) is located in endoplasmic reticulum cytoplasm [271] and it exerts its action on IGF-1R and IRS-1 [272, 273]. Absence of PTP-1B causes increased proliferation, motility and growth [272].

Internalization and ubiquitination mechanisms can alter IGF-1R functions. IGF-1R internalization is regulated by adaptor protein-2 (AP-2)-dependent endocytosis. Once in endosomal compartment, ligand dissociation exerts a regulatory role on signaling and some studies have pointed out the relevance of E-64-sensitive cysteine proteinase or cathepsin B. In addition, alterations on cathepsin L were observed in breast cancer cells [218, 274, 275]. IGF-1R ubiquitination is regulated by two E3 ligases: Nedd4 and mouse double minute 2 (MDM2). Nedd4 binds IGF-1R through the adaptor protein Grb10 and studies in mice have demonstrated that disruption in the maternal Grb10 allele induces embryo and placental overgrowth and 30% overweight compared to wild-type control [276]. MDM2 is a RING finger ubiquitin ligase that binds IGF-1R β-subunit thus starting its degradation process. MDM2 loss causes an increase in IGF-1R expression levels [277].

IGF-2R represents an important oncosuppressor for the cell. It acts as a negative regulator of IGF-2 avoiding its binding to IGF-1R or IR. Different primitive tumors display loss-of-function mutations on IGF2R gene. In addition, studies conducted in prostate cancer cells showed that IGF-2R dominant negative expression causes increased cell growth rate while IGF-2R over-expression leads to growth decrease [278].
The role of IGF-1R in tumor pathogenesis and progression in widely accepted but its value as indicator of prognosis is still not completely clarify. Clinical studies evaluating the prognostic potential of IGF-1R have reported either positive or negative associations between receptor expression levels and patient outcome in different tumor types including prostate cancer [279], non-small cell lung cancer [280]. In breast cancer no correlation was found between IGF-1R and prognosis [281] while a correlation between higher IGF-1R expression and good outcome was found in primary Ewing sarcoma patients [282].

In the last years, different studies evidenced that IR is abnormally expressed in a variety of tumor types where it mediates both metabolic and non-metabolic effects [207]. Particularly, studies demonstrated an altered splicing in tumor cells, leading to an increased ratio IR-A:IR-B that influences cell response to insulin and IGFs. IR-A is found over-expressed in carcinoma of breast, colon, lung but also rhabdomyosarcoma, leiomyosarcoma, and miosarcoma. The relevance of IR-A expression is due to its capability to bind IGF-2. IGF-2 is frequently expressed by tumor cells and its binding to IR-A is associated with stimulation of growth and cell invasion [223]. IR-B is not able to bind IGF-2 and it is mainly associated with metabolic signals. Experimental studies demonstrate that IR-A over-expression in NIH 3T3 fibroblasts or immortalized human breast epithelial cells induces a ligand-dependent transformed phenotype and that this effect is blocked by IR-blocking antibodies [283]. However, these cells were not able to induce tumor in nude mice suggesting further alterations are required for a complete transformation. The importance of IR-A in tumor progression has been demonstrated in thyroid cancer where both IR and IGF-1R are over-expressed in differentiated tumor but IR-A:IGF-1R ratio progressively increases with the differentiation loss [284]. In addition, increased IR-A expression in tumor cells favors hybrids HR-A formation thus increasing IGF-1 and 2 binding sites and conferring growth advantage to the cells [221]. In addition, IR-A/IGF-2 circuit activation has been found able to mediate resistance mechanisms toward anti-IGF-1R agents [285] thus evidencing the relevant role of IR-A in tumor.

The role of IGF-1 and IGF-2 in cancer is still controversial: a correlation between IGF-1/IGF-2 expression levels and cancer progression has been evidenced in some tumor types including liver, colon-rectal, and pancreatic carcinomas but this was not confirmed in breast cancer. Overall, the data indicate a relevant role of the ligands in tumor progression [218]. In some tumor types this proliferative stimulus is given by circulating IGF-1 while in others by a paracrine or autocrine local synthesis of IGF-1 and 2. Probably, tumors initially depend from ligands produced by the host while they become able to produce IGF-1 or IGF-2 autocrinely thus acquiring a higher malignancy
level during cancer progression. IGF-1 and IGF-2 circulating levels display a high variability among individuals especially because of polymorphisms of IGF-1 and IGF-2 gene or alterations of ligand expression regulators including IGFBPs, GH and its receptor, somatostatina, GHRH and its receptor [261]. Epidemiological studies have evidenced that higher IGF-1 circulating levels are associated with increased cancer risk. Increased IGF-1 expression and polymorphisms of IGF-1 itself or its regulators has been correlated with increased risk of prostate, lung, breast, colon-rectal carcinomas and sarcoma [286]. Several studies have demonstrated a correlation between endogenous or exogenous insulin and cancer. In the 1970’s, studies in mice showed administration of exogenous insulin caused breast and colon tumors [287]. Animal models of insulin resistance, leading compensatory insulin increase, showed same results regarding endogenous insulin effects: insulin-resistance correlates with increased colon-rectal tumor formation in rats [288] and increased proliferation of lung and colon-rectal xenografts [289]. Epidemiological studies have demonstrated an increased risk of different tumors, including colon-rectal and endometrium cancer, in individuals with higher insulin levels [290]. Recently, type II diabetes and obesity have been demonstrated to be associated with increased risk of breast, colon, liver, pancreatic and kidney tumors [207]. These studies, together with the discovery of IR-A relevance in cancer, further support the hypothesis that insulin-resistance and the consequent hiperinsulinemia represent the main link between diabetes and cancer [291]. In an insulin-resistance condition, insulin-target tissues do not respond to insulin metabolic stimuli while other epithelial tissues undergo the mitogenic insulin stimulation [292]. In this condition, insulin can bind both IR-A and IGF-1R, with low affinity. In hyperinsulinemia condition, the MAPK pathway is preferentially activated and induces cell proliferation while the PI3K pathway, regulating metabolic signals, is normally inhibited [293].

4.5 IGF-1R as a therapeutic target

Development of targeted therapeutic strategies is based on the identification of specific alterations which are able to mediate cellular oncogenic pathways. In fact, the use of target therapies is indicated for those cancer where it is possible to identify a gene or a protein able to induce malignant phenotype. Identify a “druggable” substrate represents an exceptional chance for tumor treatment and an important option to improve patient outcome. Considering its biological effects, IGF-1R has emerged as a promising target
for the development of new therapeutic approaches, which can be combined with other classical chemotherapeutics [294]. In the past decades, several IGF-1R inhibitors, including monoclonal antibodies or tyrosine kinase inhibitors, have been developed and their efficacy has been tested in a large variety of tumor types. Overall, the data show that despite satisfactory preclinical effects, clinical trials did not give the expected results and this could be due to onset of resistance mechanisms or lack of a suitable patients selection. Identification of patients who can benefit of a specific target therapy represents today one of the main challenge in the field of cancer treatment. Agents aimed at IGF-1R down-regulation include monoclonal antibodies, tyrosine kinase inhibitors but also dominant-negative proteins, antisense oligonucleotides, RNA interference have been proposed. Several monoclonal antibodies have been developed to target IGF-1R. Monoclonal antibodies bind the extracellular domains of the receptor and block ligand binding. In addition, antibodies are able to induce IGF-1R down-regulation by promoting receptor internalization. The first available anti-IGF-1R antibody was αIR3. In 1989, Arteaga et al. demonstrated its efficacy as inhibitor of human breast cancer cells in athymic mice growth [295]. Some anti-IGF-1R antibodies are able to down-regulate IR, while being IGF-1R specific, due to down-regulation of IGF-1R:IR hybrids or to endocytosis of IR in close proximity to IGF-1R in membrane lipid drafts [296]. Fully human antibodies have been developed and tested in preclinical and clinical trials. Anti-IGF-1R monoclonal antibodies entered into clinical trials include CP-751,871 (figitumumab; Pfizer) and AVE1642 (ImmunoGen Inc. and Sanofi). CP-751,871 is a fully human IgG2 blocking binding of IGF-1 to its receptor, IGF-1-induced receptor autophosphorylation and IGF-1R down-regulation [297, 298]. Its efficacy has been tested in phase I trials in different tumors including ES, prostate cancer, and multiple myeloma demonstrating to be well tolerated but with low effectiveness especially alone. AVE1642 is a humanized version of the anti-IGF-1R antibody EM164 [299]. Clinical trials have been performed to test its efficacy alone or in combination in a variety of solid tumors. Overall, data indicate again that the agent is well-tolerated but with partial responses especially in sarcomas [300]. Biomarkers for sensitivity to these agents remain an urgent need. Several compounds have been design to inhibit the tyrosine kinase activity of IGF-1R. From this point of view, complications are due to the high homology rate between IGF-1R and IR particularly in the tyrosine kinase domain (85%) and the ATP binding cleft (100%). Small molecule inhibiting the kinase domain include NVP-AEW541 and NVP-ADW742 (Novartis) appearing to be equipotent for IGF-1R and IR inhibition in vitro but specific for IGF-1R in intact cells [301, 302]. These agent displayed a good tumor growth inhibition in models of fibrosarcoma, myeloma, and ES but development of
these agents was let down by toxicity. Inhibitors of ATP binding cleft lack IGF-1R specificity while inhibitors of substrate phosphorilation show greater potential. In particular, the cyclolignan picropodophyllin showed a great specificity for IGF-1R as it inhibits tyrosine phosphorylation of Y1136 in the activation loop of kinase domain. This agent showed a good potential in vitro. This class of compounds include OSI-906 (Astellas Pharma), a novel ligand-dependent autophosphorylation inhibitor of both IGF-1R and IR which show a good potential in those tumors expressing the two receptors. OSI-906 demonstrated good efficacy in preclinical studies and it is currently in clinical testing especially in combination with other drugs such as everolimus in colorectal cancer [303], doxorubicin in breast tumor cells [304]. In addition, biomarkers were found predicting response to OSI-906 in hepatocellular carcinoma cell lines [305] or in colorectal cancer [306]. Several phase I and II clinical trials are ongoing.

Dominant-negative proteins interfere with the wild-type protein by direct binding or competition for binding substrates. The first described dominant-negative is 486/STOP, holding an intact ligand-binding domain [307]. Its effects have been tested in experimental models of breast, pancreatic, lung, colon tumors where inhibits migration and metastases. Translation of this approach to clinical practice is limited by low efficiency of delivery and stability.

Antisense oligonucleotides approach involves introduction into the cells of antisense RNA or antisense oligonucleotides (ASOs) binding to a complementary mRNA target. Gene expression is inhibited by RNase H-mediated mRNA cleavage or translation block. Experimental studies demonstrated that antisense against IGF-1R cause growth inhibition and apoptosis induction in vivo. Use of oligoantisense in clinic is limited by low cellular up-take, non specific toxicity. Topical application or ex vivo transfection represent two putative options to overcome these limitations. In the last case, patients treated with ex vivo approaches display a clinical amelioration in 60% of cases [308] confirming the benefit of an anti-IGF-1R treatment.

RNA interference technique known since 1998 as a process in which long double-stranded RNAs (dsRNAs) are cleaved by Dicer ribonuclease enzyme into short 21-23 nucletide fragments called short interfering RNAs (siRNAs). siRNAs are incorporated into a multi-protein RNA-inducing silencing complex (RISC) in which the antisense siRNA target the cleavage of a complementary mRNA [309]. As dsRNAs can induce apoptosis in mammalian cells, through stimulation of interferon expression, direct introduction of siRNAs enabled the use of this tool in research cell biology. Molecular interaction between siRNA and mRNA depends on the accessibility of the target as it was demonstrated in breast cancer cell [205]. In vivo use of siRNA is limited by poor cellular uptake and susceptibility to degradation which could be reduced by
incorporation within nanoparticles or conjugation with cholesterol. siRNA against IGF-1R showed down-regulation of the target and decrease of tumor cell growth in pancreatic cancer [310], and colon cancer cells [311].

### 4.6 IGF system in Ewing sarcoma

The importance of IGF system in ES was first described by Scotlandi et al. in 1996. The authors demonstrated the presence of a unique, autocrine loop mediated by IGF-1R in ES and PNET cell lines due to an autocrine production of IGF-1. Moreover, blockage of the IGF-1R-mediated circuit by anti-IGF-1R αIR3 monoclonal antibody inhibits ES/PNET cells growth via increased apoptosis, decrease of proliferation rate as well as soft-agar growth and migration upon chemotactic stimulus [312]. Relevance of IGF-1R in ES transformation was then demonstrated by Toretsky et al. with an *in vitro* study performed on fibroblasts derived from IGF-1R knockdown or wild type mice. Co-presence of both EWS-FLI1 and IGF-1R is required for cell transformation as lack of one of the two factors prevents soft-agar growth. In addition, EWS-FLI1 alters IGF-1R signaling as demonstrated by higher levels of phosphorilated IRS-1, one of the main mediators of IGF-1R pathway, compared to cells non expressing the fusion gene [313].

Subsequently, a study focused on the identification of EWS-FLI1 target genes demonstrated that EWS-FLI1 silencing induces expression of IGFBP-3, a key regulator of proliferation signal mediated by IGF-1. ChIP analysis showed that EWS-FLI1 binds *IGFBP-3* promoter and represses its transcription. In this landscape, inhibition of this protein represents a crucial event in ES tumorigenesis [78]. Confirmation of perturbing effects of EWS-FLI1 on IGF system were given by other studies performed on mesenchymal progenitors: EWS-FLI1 induces expression of IGF-1 and inhibits IGFBP-3, IGFBP-5 and IGFBP-7 [66, 78, 314]. Considering the importance of the IGF-1R/IGF-1 loop in ES pathogenesis, down-stream mediators status was investigated in ES cells demonstrating that MAPK and PI3-K pathways appeared to be constitutively activated. Utilization of specific inhibitors of MAPK and PI3-K, PD98059 or U0126 and LY294002, respectively, impaired ES cell growth in monolayer and soft-agar basal conditions inducing $G_1$ blockage or affecting cell survival. In addition, MAPK inhibition reduced migratory ability of ES cells and increased chemosensitivity to doxorubicin, a leader drug in ES treatment [315].

Considering the relevance of IGF-1R in ES cell growth, efficacy of its inhibition was tested *in vitro* and *in vivo*. IGF-1R blockage in ES causes inhibition of cancer cell
proliferation, survival, and anchorage-independent growth, inhibits tumorigenesis, tumor invasion and metastasis, and sensitizes cancer cells to chemotherapy and radiotherapy [206]. In vivo, αIR3 monoclonal antibody treatment was shown to induce complete tumor regression in 50% of athymic mice [316].

### 4.7 IGF system in prostate cancer

In the prostate, IGF-1R plays a critical role in normal gland growth and development, as well as in cancer initiation and progression [317]. First evidences regarding a putative role of IGF system in this tumor type came from epidemiological studies showing that higher IGF-1 serum concentration as well as decreased circulating IGFBP-3 correlated with increased risk of PCa [318]. Subsequently, several in vitro and in vivo studies were performed to assess IGF system role in PCa. Particularly, significance of prostate stromal IGF-1 in PCa development was confirmed by Kawada et al.: conditioned medium of prostate stromal cells, being rich in IGF-1, induced phosphorilation of IGF-1R and increased growth of PCa cell lines. Furthermore, only chemical IGF-1R inhibitors suppressed the prostate stromal cells-induced growth in PCa cells [319]. It was concluded that stromal IGF-1 accelerates tumor growth in prostate. IGF-1R and IGFBPs were found expressed in cell lines encouraging researchers to study the putative role of IGF-1R in regulating growth, survival and metastases. A study conducted on 54 samples of primary PCa showed that IGF-1R was significantly up-regulated at protein and mRNA levels compared to benign prostatic epithelium. In addition, IGF-1R expression was maintained in metastasis samples [320]. This result is in accordance with another study showing that intensity of IGF-1R immunostaining increased from benign prostatic tumor over PIN to carcinoma [321]. The interaction between androgens and IGF system has been considered too and some studies demonstrated that androgens induce a selective up-regulation of IGF-1R in PCa cell lines via c-Src/ERK/cAMP–response element-binding protein (CREB) activation that stimulates IGF-1R promoter. Through this mechanism, androgens increase cell proliferation and invasiveness in response to IGF-1 thus contributing to the progression to castration-resistant PCa [322, 323].

However results in the field of IGF system role in PCa are still controversial. Indeed, some studies did not find any significant differences between IGF-1R expression levels between normal and tumor tissues [324, 325] while others evidenced a correlation between IGF-1R loss and malignancy. In particular, data from an in vitro study reported a correlation between progression toward a metastatic stage and reduction of IGF-1R
expression [326]. An in vivo study performed by Sutherland et al. showed that in conditional (Cre-loxP) prostate-specific IGF-1R knockout mouse model, IGF-1R abrogation caused cell proliferation, hyperplasia and emergence of aggressive PCa when p53 activity was compromised [327].

5. CD99 molecule

5.1 MIC2 gene and CD99 protein

CD99 antigen was identified in 1979 thorough 12E7 monoclonal antibody as highly expressed in human lymphocytic leukemia cells [328]. CD99, also known as E2, is a 32 kDa glycoprotein codified by MIC2 gene which is located on pseudo-autosomal region of sex chromosomes: X (Xp22.33-Xpter) and Y (Yp11-Ypter). The gene is 50kb and it is orientated towards the centromere, MIC2 contains 10 exons that are smaller than the average of mammalian genes. In addition, evolutionary studies indicate that the gene can be detected by DNA hybridization only in primates [329]. MIC2 does not belong to any known gene family but correlates with two genes located in the same pseudo-autosomal region: PDBX and MIC2R. PBDX codifies for the Xg<sup>a</sup> blood group antigen sharing a 48% homology with CD99 [330] while MIC2R (MIC2-related) is related to exons 1, 4 and 5 of MIC2. Transcripts from the MIC2R locus have been detected in all human tissues but none of them contains a significant open reading frame making the functional role of MIC2R still unknown [331].

CD99 gene encodes two distinct proteins as a results of alternative splicing process: a wild-type full length CD99 or type I (CD99wt) and a truncated form or type II CD99 (CD99sh). Particularly, the CD99sh was identified in the process of screening a human thymus λgt11 cDNA library by Hahn JH et al. in 1997. The CD99sh transcript contains a 18-bp insertion at the boundary of exons 8 and 9 of MIC2 gene introducing an in-frame stop codon that generates truncated polypeptide [332]. The resulting protein contains 160 amino acids compared to the 185 amino acids of CD99wt. Conservation of
the two isoforms in primates suggests that both of them hold an important role but regulatory mechanisms underlying splicing mechanisms are still unknown.

Sequence analysis of CD99wt cDNA suggests that CD99 contains a 100 amino acids extracellular domain glycosilated with O-linked sugars, a putative transmembrane domain of 25 amino acids and a 38 amino acids cytoplasmic domain. Biochemical studies showed that CD99wt is highly sialylated and glycosylated, it carries no N-linked sugar residues while carries O-linked oligosaccharides. Post-translational modifications induce a molecular mass reduction going from 32 kDa to 28 kDa and 18 kDa after neuraminidase and O-glycanase treatment, respectively [333]. The 32 kDa molecule displays a certain similarity with collagen and other glycoproteins including CD43 and CD34 involved in cell adhesion processes [334]. More recently, multidimensional heteronuclear NMR and CD spectroscopy approaches have pointed out that CD99 cytoplasmic domain is composed of an α-helical conformation forming a symmetric dimer in the presence of the transmembrane domain. Consequently, no secondary structure element seems to be present [335].

5.2 CD99 expression in normal and tumor tissues

Immunohistochemical analysis evidenced that CD99 is ubiquitously expressed in normal cells and, particularly, an elevated CD99 expression has been found in ependymal cells of brain and spinal cord, pancreatic islets, Leydig and Sertoli cells, ovarian granulose cells and endothelial cells. Functional role of CD99 in these cells is unknown while more information are available regarding its role in hematopoietic system. In 1994, Dworzak et al. reported that CD99 is expressed in cells of all leukocyte lineages and that its expression is correlated with maturational stages. Particularly, the authors evidenced a general highest density of CD99 in the most immature stages of the lymphocytic and granulocytic lineages. In the B-lineage cells, CD99 expression is observed in the earliest maturational stages characterized by co-expression of CD34 and CD10 antigens but is lost during maturation. Similarly, a remarkable CD99 expression was observed in immature T-cells while low expression was detected upon differentiation [336]. In the erythrocytes, MIC2 product displays a quantitative polymorphism co-regulated with Xgα blood group antigen. This co-expression is supposed to be regulated at transcriptional level [337].

In addition to its physiological function, CD99 has a role in pathology and, particularly, in cancer. Several evidenced demonstrate its role in acute lymphoblastic leukemia, embryonal rhabdomyosarcoma, synovial sarcoma, mesenchymal condrosarcoma, gastric
and ependimal tumors, in Ewing sarcoma and PNET. Particularly, in Ewing sarcoma and PNET, CD99 represents a diagnostic biomarker together with specific chromosomal aberrations [338].

Considering that no antibody is able to specifically target CD99sh, RT-PCR represents the best technique for CD99 isoforms discrimination. Overall, studies conducted in normal and pathological tissues evidenced that CD99sh is expressed at lower levels compared to CD99wt but its production may be up-regulated during specific stages of cell differentiation, physiologic conditions or locations in the cells [332]. Accordingly, it has been described that in immature T-cell lines CD99 is expressed as heterodimers composed of a long chain associated with a short variant. During cell differentiation, CD99 expression levels decrease putatively because a reduced expression of CD99sh [339]. In addition, Lee et al. pointed out an association between CD99 subtypes expression and neural differentiation in Ewing sarcoma cells. The authors found that CD99sh was reduced upon differentiation while no difference was found in CD99wt expression levels. These data suggest that in this tumor type CD99sh acts as a negative regulator of neural differentiation [340].

5.3 CD99 function in normal and tumor tissues

Physiological functions of CD99 (wild type form) are not completely clear especially because its ligand is still unknown and the protein structure still remains not completely defined. Use of monoclonal antibodies directed against CD99 allowed identification of some functions carried out by CD99. The first studies were performed in hematopoietic tissues where CD99 function appears particularly correlated with cellular differentiation degree. First evidences in T-lymphocytes pointed out that CD99 presence on the surface was involved in spontaneous rosette formation with erythrocytes. As monoclonal antibodies directed against CD99 blocked rosette formation [341], the authors demonstrated that CD99 mediates T-cell adhesion events. In addition, CD99 activity and density was found related with differentiation stage. Particularly, stimulation with MAbs induced homotypic cell aggregation and apoptosis in CD4+ and CD8+ corticothymocytes but not in other T cells [342] suggesting a role in cellular differentiation. A role of CD99 was also described in peripheral blood lymphocytes as anti-CD99 antibodies treatment enhanced T cell proliferation in the presence of anti-CD3 stimulation and resulted in a marked expression of T-cells activation markers like CD69, CD40L [343]. Some studies pointed out that CD99 modulates adhesion of T cells to the vascular endothelial cell wall during leukocyte extravasation events.
Particularly, E2 MAbs stimulation was shown to increase affinity/avidity for α4β1 integrins of peripheral T cells with CD4+ memory, but not CD4+ virgin T cells, on endothelial vascular cell-adhesion molecule 1 (VCAM-1) [344]. In addition, some studies evidenced the importance of CD99 expression on endothelial cells for monocytes transendothelial migration [345].

In order to investigate physiologic relevance of CD99 in vivo, Bixel et al. cloned a mouse cDNA coding for a protein 45% identical in its sequence with human CD99. Antibodies against this mouse homolog were used in leukocytes and the results showed that the treatment induced blockade of cell aggregation, inhibition of transendothelial migration and inhibition of recruitment on in vivo-activated T cells into inflamed skin and edema formation [346].

Some studies evidenced a link between CD99 and osteoblast lineage. In particular, in a study performed on osteosarcoma cells, MIC2 gene was demonstrated to be a target of Cbfa1, a key transcription factor of osteoblast differentiation. Overexpression of Cbfa1 was induced in SaOs-2 through transfection of a dominant negative mutant of Cbfa1(ΔCbfa1) and this procedure allowed the identification of 4 genes putatively under the control of Cbfa1. Between them MIC2 was found as strongly overexpressed in mutant cells compared to wild type cells. It is still unclear whether Cbfa1 directly regulates transcription of MIC2 or through a signaling pathway.

Several studies have been carried out to elucidate the role of CD99 in pathology and, particularly, in cancer. Overall, data regarding CD99 in tumor point out a dual role of this molecule, acting as an oncogene or an oncosuppressor depending on the cellular context [347].

High levels of CD99 were found particularly in ES, where it is also used as a relevant diagnostic tool [338]. CD99 stimulation with monoclonal antibodies is reported to induce cell death and homotypic adhesion in vitro as well as to reduce cell growth and metastasis in vivo [348]. More recently, Rocchi et al. demonstrated, both in vitro and in vivo, that silencing of CD99 correlates with an increased neural differentiation as demonstrated by neurite-like outgrowth and higher expression of neural differentiation markers such as beta-III tubulin and H-neurofilament [94]. In addition, CD99 was found as a putative target gene of EWS-FLI1 as demonstrated by ChIP assay results showing the recruitment of FLI1 on CD99 promoter in ES cells [94]. Considering CD99 is easily accessible, it is expressed in all cases and mediates malignancy in ES, it represents a suitable target for specific antibodies with putatively clinical application. With this purpose, a nonimmunogenic human recombinant monospecific antibody against CD99 (dAbd C7) has been recently developed and its efficacy has been tested both in vitro and in vivo. dAbd C7 gave satisfactory results in cell lines and in mice when combined with
doxorubicin without affecting viability or differentiation of normal human mesenchymal stem cells [349].

Noteworthy is CD99 role in acute lymphoblastic leukemia as CD99 is found strongly expressed in tumor cells compared to normal counterpart [350]. In addition, in this tumor type CD99 evaluation represents a putative marker of minimal residual disease [351].

On the opposite side, an oncosuppressive role for CD99 has been postulated in other tumors. In lymphomas, *in vitro* down-regulation of CD99 generates B lymphocytes with Hodgkin and Reed-Sternberg phenotype [352]. In pancreatic endocrine carcinomas, CD99 is lost in malignant lesions compared to the benign and CD99 loss correlates with a worse prognosis [353, 354]. In gastric adenocarcinoma, down-regulation of CD99 was found associated with dedifferentiation of tumor cells suggesting a role for this protein in tumorigenesis or tumor progression [355]. Studies conducted by Pelosi et al. demonstrated the relevance of CD99 in gastrointestinal and pulmonary neuroendocrine tumors evidencing a correlation between loss of CD99 positive cells and local invasion or distant metastases [356] as well as an association between loss of CD99 and occurrence of nodal metastases in patients [357].

CD99 is reported to act as an oncosuppressor in osteosarcoma as demonstrated by Manara et al. In this study, the authors demonstrated that CD99 expression is low or absent in osteosarcoma cells and patient specimens compared to normal counterparts. As a confirmation, CD99 forced over-expression significantly reduced resistance to anoikis, inhibited growth in anchorage-independent conditions and migration as well as abrogated tumorigenic and metastatic ability [358]. In addition, a recent study evidenced that forced CD99 expression suppresses osteosarcoma cell migration through a marked inhibition of ROCK2/ezrin axis [359].

Sporadic data pointed also out a putative oncosuppressive role of CD99 in prostate cancer. Forced over-expression of CD99 in prostate cancer cells causes an inhibition of soft-agar growth as well as migration capability. Studies in mice confirmed that cells over-expressing CD99 display a decreased tumor incidence and a reduced formation of lung or bone metastases [347].
Aim of the study

Aberrant expression of *ETS* genes due to chromosomal translocations has been described as a common feature of different tumor types including Ewing sarcoma and prostate cancer. The ETS family includes several transcription factors sharing high sequence homology in the DNA binding domain and regulating genes involved in cancer initiation and progression. In Ewing sarcoma, EWS-ETS fusion proteins act as aberrant transcription factors modulating the expression of a variety of known target genes and driving pathogenesis of this tumor. In prostate cancer, the TMPRSS2-ETS fusion gene, resulting in over-expression of an ETS member, represents an early event in cancer progression but less information are available regarding the target genes network dysregulated by this rearrangement. In this study, the impact of TMPRSS2-ERG on two well documented targets of EWS-FLI1, including components of the insulin-like growth factor system and the CD99 molecule, was evaluated. The aim of this study was to identify common or distinctive ETS-related mechanisms which could be exploited both at biological and clinical level. From the biological point of view, identification of target genes for oncogenic ETS transcription factors between different tumors may lead to a broader understanding of more general mechanisms underlying malignant transformation. From the clinical standpoint, fusion genes and definition of their cross-talk may allow identification of molecules with a putative relevance at prognostic or therapeutic level.
Materials and Methods

CELL LINES

Prostate cancer cell lines PC-3, LNCaP, DU-145, VCaP were obtained from the American Type Culture Collection (ATCC). 22RV1 prostate cancer cell line was purchased from Sigma Aldrich. Immortalized non-malignant prostate cell line RWPE-1 and stable transfectants RWPE-1_tERG or RWPE-1_empty vector were kindly provided by Dr. Gambacorti-Passerini, University of Milano-Bicocca. ES cell lines TC-71 and 6647 were kindly provided by T.J. Triche (Children's Hospital, Los Angeles, CA). LAP-35 EWS cell line was obtained in the Experimental Oncology Lab, Rizzoli Institute (Bologna). PC-3, LNCaP, DU-145, TC-71, 6647 and LAP-35 cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) (Lonza). 22RV1 cells were maintained in RPMI 1640 (Gibco) while VCaP cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) implemented with L-glucose and bicarbonate. RWPE-1 and transfectant cells were maintained in keratinocyte-serum free medium supplemented with epidermal growth factor and bovine pituitary extract (Life Technologies Inc.). Culture medium of stable transfectants RWPE-1_tERG or RWPE-1_empty vector cells was implemented with G418 250μg/ml for selection maintenance. IMDM, RPMI and DMEM media were supplemented with 10% inactivated Fetal Bovine Serum (FBS) (Lonza) and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. All cell lines were tested for mycoplasma contamination every 3 months by MycoAlert mycoplasma detection kit (Lonza) and were recently authenticated by STR PCR analysis using genRESVR MPX-2 and genRESVR MPX-3 kits (Serac). The following loci were verified for PCa cells: D3S1358, D19S433, D2S1338, D22S1045, D16S539, D18S51, D1S1656, D10S1248, D2S441, TH01, VWA, D21S11, D8S1179, FGA, SE33. For ES cells the following loci were verified: D16S539, D18S51, D19S433, D21S11, D2S1338, D3S1358, D5S818, D8S1179, FGA, SE33, TH01, TPOX VWA.

CLINICAL PROSTATE SPECIMENS

Formalin fixed and paraffin-embedded (FFPE) blocks corresponding to PCa patients were retrieved from the archives of the Biobank of the Fundación Instituto Valenciano
de Oncología according to the following criteria: specimens obtained from radical retropubic prostatectomies from 1996 to 2002 and no history of previous treatment for PCa (including androgen deprivation therapy or chemotherapy prior to surgery). 270 cases were identified to meet these criteria. All patients gave written informed consent for tissue donation for research purposes before tissue collection, and the study was approved by FIVO’s Institutional Ethical Committee (ref. number, 2010-19). Clinical data were reviewed from clinical records and stored in a PCa-specific database. Patient characteristics, including the T2E fusion gene status, and demographics are shown in Table 1. Combined Gleason score was uniformly regarded by the same uro-pathologist (Ana Calatrava from Fundación Instituto Valenciano de Oncología, Valencia, SPAIN), who also certified high-density cancer areas in haematoxylin and eosin stained slides to ensure a purity of at least 75% of cancer cells. For comparative and calibration purposes, we also analyzed 10 samples of normal prostate tissue obtained from patients operated of radical cystectomies without pathological evidence of prostatic disease. T2E gene fusion status was determined by RT-PCR and fluorescent in situ hybridization (FISH) as described by and quantitative RT-PCR. Follow-up of the retrospective series ranged from 2 to 189 months (median 96 months). Biochemical progression was defined as serum PSA greater than 0.4ng/ml during follow-up and clinical progression was defined as local (prostatic fossa), regional (lymph nodes) or distant (metastasis) progression.

DRUGS

Anti-IGF-1R drugs were kindly provided by: ImmunoGen Inc. (AVE1642, a humanized version of anti-IGF-1R EM164 antibody), Pfizer (CP-751,871/Figitumumab), and Novartis (NVP-AEW541). Abiraterone acetate (S1123) and Cabazitaxel (S3022) were purchased by Selleckchem. Trabectedin was provided as lyophilized formulations and as clinical preparation by PharmaMar S.A., Colmenar Viejo, Madrid, Spain. OSI-906 (Linsitinib) was purchased by Selleck Chemicals. Doxorubicin (DXR) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Working dilutions of all drugs were prepared immediately before use.
GENE EXPRESSION ANALYSIS

Cell line analysis

Cell lines total RNA (2 mg) was extracted with TRIzol (Invitrogen), a monophasic solution of phenol and guanidine isothiocyanate, and purified by precipitation with isopropanol. Oligo dT primers (Applied Biosystems) were used to reverse transcribe RNA with a 260/280 nm absorbance ratio of 1.5-2. Quantitative Real-Time PCR was performed on ABI Prism 7900 (Applied Biosystems) using TaqMan or SYBR Green assays (Applied Biosystems). Following predesigned TaqMan assay for target gene was used: IGF-1R (Hs00181385_m1), CD99 (Hs00365982_m1). Following SYBR Green assays for target genes were used: IR (forward 5’-CGTGGAGGATAATTACATCGTGT-3’ and reverse 5’-TGGTCGGGCAAACCTTTCT -3’, 167bp), IGF-1 (forward 5’-TCGCATCTCTTCTACCTGGCGCTGT-3’ and reverse 5’-GCAATACATCTCCAGCCTCTAGA-3’, 240bp), IGF-2 (forward 5’-GACCGCGCTTCTACTTCAG -3’ and reverse 5’-AAGAACTTGCCCACGCGGTAT -3’, 203bp). Primer Express software (Applied Biosystems) was used to design appropriate primer pairs and probe for reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5’-GAAGGTCAGGTGTCGAGTCGTC-3’, reverse 5’- GAAGATGATGATGATGATGATGAT -3’ and probe 5’- CAAGCTTCCGCGTTCTCTCCCTCC -3’. Two replicates per gene were considered. Relative quantification analysis was performed on 2^-ΔΔCt method. Absolute quantification assay for the measurement of total IGF-1R was performed by Dr. Roberta Malaguarnera accordingly to the procedure published in 2011 [360].

Clinical specimens analysis

Isolation of RNA from three sections of 10μm paraffin-embedded tissue was performed using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion) following providers’ specifications. RNA with a 260/280 nm absorbance ratio of 1.5-2 was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer’s indications. Clinical samples were analyzed using ABI 7500-Fast Thermocycler Sequence Detection System (Applied Biosystems), according to manufacturer’s instructions. Predesigned TaqMan probes for target genes were used: IGF-1R (Hs00181385_m1), IR (Hs00961560_m1), IGFBP-3 (Hs00426287_m1), IGF-1
(Hs 00153126_m1), IGF-2 (Hs04188276_m1), CD99 (Hs00365982_m1), T2E (Hs03063375_ft). For endogenous control β2-microglobulin: (Hs99999907_m1) was used (Applied Biosystem).

cDNA from normal human prostate samples was used as calibrator for comparative analysis of PCa cases. Two replicates per gene were considered. Relative quantification analysis was determined using the mean value of the control samples and following the 2-ΔΔCT method.

**CHROMATIN IMMUNOPRECIPITATION (ChIP)**

For ChIP assay cells plated in 60 or 100 mm Ø dishes were washed twice with PBS and crosslinked with 1% formaldeyde at 37°C for 10 minutes. Cells were then washed with fresh PBS, collected, resuspended in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1) and kept in ice for 10 minutes. Cells were then sonicated four times for 10 seconds at 30% of maximal power and collected by centrifugation at 4°C for 10 minutes at 14000 rpm. Supernatants were collected and diluted in IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM NaCl) and precleared with sonicated salmon sperm DNA/protein A agarose (UBI) for 1 hour at 4°C. Precleared chromatin was immunoprecipitated for 12 hours with anti-ERG-1/2/3 (C-17, Santa Cruz Biotechnology) or anti-FLI1 antibodies (C-19, Santa Cruz Biotechnology). Salmon sperm DNA/protein A agarose was added and precipitation was continued for 4 hours at 4°C. Pellet was collected and the precipitates were washed sequentially for 5 minutes with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), Wash C (0.25M LiCl, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1), and twice with TE buffer (10mM Tris, 1mM EDTA). The immune complexes were eluted with elution buffer (1% SDS, 0.1M NaHCO₃). The eluates were reverse crosslinked by heating at 65°C for 12 hours and digested with proteinase K (0.5mg/ml) at 45°C for 1 hour. DNA was obtained by phenol/chloroform/isoamylalcohol (25:24:1) extractions. Yeast tRNA was added to each sample and DNA was precipitated with EtOH for 12 hours at 4°C and resuspended in TE buffer. Samples were quantified with Nanodrop. Quantitative RT-PCR was performed with custom primers flanking Ets-containing target promoters fragment. IGF-1R promoter was evaluated by Real-Time PCR using the following custom TaqMan assay: forward 5’-AGGAGGAGGAGGAGGAGGAG-3’, reverse 5’-GCAGTTCGCAAGATCGCC-3’ and probe 5’-TTGACTCCGCGTTTCTGCCCCCTCG-
3’. For the TaqMan assay design TFSEARCH - Searching Transcription Factor Binding Sites, version 1.3 free website was used for the prediction of ETS binding sites in the promoter of IGF-1R gene and the sequence spanning from 1041bp to 1051bp was identified as the best. Beacon Designer 4 software was used for the design of the assay spanning from 1005bp to 1114bp. PIM-1 promoter fragment containing ETS consensus sequence was used as ERG immunoprecipitation positive control [174] by Real-Time PCR using the following SYBR Green assay: forward 5’- GTGCTAGGCGAGTGGGAACAAACTG-3’ and reverse 5’- AATGACCACAAATTCCACCTCCTGAG-3’. CD99 and TGFβR2 promoter fragments containing ETS consensus sequence were evaluated with the following SYBR Green assays: CD99 promoter forward 5’- TTGTTAAGTGTGGGAAGGGC-3’ and reverse 5’- CTGCTCACCTCAGGGAGTTC-3’ (417bp), TGFβR2 promoter 5’- GTGTGGAGGGCGGTGAGGGGC-3’ and reverse 5’- GAGGGAAGCTGCACAGGAGTCCGGC-3’ (285bp). For quantitative PCR (qPCR) data are calculated with the following formula: % of recruitment = 2ΔCt x input chromatin percentage, where ΔCt = Ct (input) - Ct (ETS IP) in accordance to Frank SR et al [361].

**IMMUNOHISTOCHEMISTRY**

PCA specimens were incorporated in 11 tissue microarrays (TMA). Two or three representative areas (1 mm in diameter) of each tumor were selected for TMA production by first examining hematoxylin and eosin-stained prostatectomy tumor slides and then sampling tissue from the corresponding paraffin blocks. A tissue microarray instrument (Beecher Instruments) was used for TMA assembly. From TMA blocks, 3-μm-thick sections were immunostained using rabbit anti-human IGF-1Rβ C-20 sc-713 (Santa Cruz Biotechnology), anti-human IRβ C-19 sc-711 (Santa Cruz Biotechnology), mouse anti-CD99 or anti-human ERG clone EP111 polyclonal-Ab (Dako). Percentage of positive cells and cytoplasmic staining intensity were scored semiquantitatively, forming four groups (from 0 to 3). Cases were scored as low expression when staining intensity was between 0 and 1, and high expression when intensity was 2 and 3.
In Vitro Assays

To assess drug sensitivity, MTT assay (Roche) was used according to manufacturer’s instructions. Cells were plated into 96 well-plates (range 2,500-10,000 cells/well). After 24 hours, various concentrations of AVE1642 (0.01-50 µg/ml), NVP-AEW541 (0.03-5 µM), CP-751,871 (0.5-500 µg/ml), trabectedin (0.3-3 nM), OSI-906 (0.3-3 µM) or Abiraterone acetate (1-100µM) were added and cells exposed to these drugs for up to 72 hours. Short interfering RNA knockdown of ERG was performed with siRNA from Thermo Scientific Dharmacon: siGENOME_siRNA (D-003886-01). siGENOME_non targeting_siRNA was used as control (D-001210-01-05). siRNA was transfected in VCaP cells using siport NeoFX transfection agent (Life Technologies Inc.) according to manufacturer’s instructions. Silencing was assessed after 48, 72, 96 and 120 hours from transfection. VCaP cells were pre-treated with ERG siRNA (100nM) for 48 hours and then exposed to CP-751,871 (0.01-1 µg/ml), NVP-AEW541 (0.2-2 µM) or Abiraterone (3-30 µM) for 72 hours. Cell growth was assessed with Trypan Blue. ERG and IGF-1R protein expression was investigated upon 72, 96 and 120 hours of Abiraterone treatment (3-10 µM). Short interfering RNA knockdown of EWS-FLI1 was performed with Lipofectamine (Invitrogen) following the manufacturer’s protocols. siRNA was used at concentrations of 75 or 100 nM for 24 hours and siRNA sequences are the following: CACCCACGTGCCTTCACAC targeting the 3’ portion of FLI1 (IDT). On-TargetPlus NonTargeting Pool (Thermo Scientific Dharmacon) was used as scrambled control. For combined treatments, cells were treated for 72h with drugs alone or combined in fixed ratios. VCaP cells were treated for 72 hours with varying concentrations of CP-751,871 (1-100 µM) and Abiraterone (1-100 µM) or Cabazitaxel (0.003-0.3 µM). In Trabectedin and OSI-906 combination experiments, ES cells were treated in fixed ratio 1:1000 while PCa cells were treated in fixed ratio 1:100.

Western Blotting

Proteins were collected from cells plated in 60 or 100 mm Ø dishes with a sub-confluence status. Cell lysis was performed on ice with UPSTATE buffer for phosphorilated proteins (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 1mM EDTA, 0.25% sodio deossicolato, 1mM NaF) upon addition of proteases inhibitors (1:100): aprotinin (10µg/ml), leupeptin (0.1mM), PMSF (1mM), sodium orthtovanadate (0.2mM). After 30 minutes incubation, lysates were centrifuged and proteins from
solution fraction were collected. Protein concentration was obtained upon dilution in Protein Assay (Bio-Rad) (1µl in 999µl) and spectrophotometer reading compared to a standard curve. Equivalent amounts of total cell lysates were separated by 10% SDS-PAGE under denaturing conditions and transferred onto nitrocellulose membrane (Bio-Rad). Ponceau (Sigma-Aldrich) staining was used to evaluate transfer quality. Membranes were blocked for 1 hour at room temperature with milk diluted in TBST (10 mM Tris-HCl ph 7.4, 150 mM NaCl e 0.1% Tween20) and than incubated overnight with the following primary antibodies: anti-IGF1-Rβ, anti-Irβ, anti-GAPDH, anti-LAMIN B, anti-ERG-1/2/3, anti-FLI1 (Santa Cruz Biotechnology), anti-AR (Cell Signaling Technology), anti-actin, anti-CD99. Membranes were then incubated with secondary anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Amersham) and revealed by ECL Western blotting detection reagents (GE Healthcare).

STATISTICAL ANALYSIS

Differences among means where analyzed using two-sided Student’s t test. Drug-drug interactions combination index (CI) was calculated with the isobologram equation of Chou-Talalay [362] by using CalcuSyn software (Biosoft). IC50 values were determined using CalcuSyn software (Biosoft). Correlations analysis were performed using Fisher’s exact test. Kaplan-Meier proportional risk log rank test was applied for survival curves. BPFS and PFS were considered individually from the date of surgery to the date of event. Univariate predictors of outcome were entered into a Cox proportional hazard model using stepwise selection to identify independent predictors of prognosis, considering the 95% CI.
Table 2. Clinicopathologic features of PCa patients evaluated for IGF system components and CD99 expression by qRT-PCR or by IHC.

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<th>IHC (n = 243)</th>
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<td></td>
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<td>%</td>
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<tr>
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<tr>
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<td>65,9</td>
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SP, specimen; cT, clinical stage; PSA, prostatic specific antigen; pN, lymphnode pathological stage
*Lymphadenectomy was limited to the obturator fossa in most of the cases at the inclusion period
**IHC ERG expression was not detectable in 24/243 and negative in 85/219 cases (39%)
Results

1. TMPRSS2-ERG and IGF system

1.1 Analysis of IGF system main components expression in PCa cell lines

A panel of six prostate cell lines, including malignant VCaP, DU-145, PC-3, LNCaP, 22RV1 and the non-malignant RWPE-1 cells was analyzed for the basal expression of different components of the IGF system. The panel of cell lines was characterized by different expression levels of androgen receptor (AR) and the TMPRSS2-ERG (T2E) gene fusion presence. VCaP cell line was the only one characterized by expression of T2E as limited models of PCa cell lines constitutively expressing T2E are available. As shown in figure 7, T2E presence corresponds to a high expression of ERG transcription factor.

![Figure 7](image.png)

**Figure 7.** Western blotting analysis of AR and ERG1/2/3 expression in a panel of PCa cells. GAPDH is shown as a loading control.

No IGF-1 or IGF-2 expression was detected in cell lines confirming a paracrine activation of the IGF pathway, putatively exploited by the stroma, in this tumor. As shown in figure 8, IR was found generally higher in PCa cells with respect to normal
cells. IGF-1R expression was found lower in PCa cells compared to RWPE-1 cell line with the only remarkable exception of VCaP cells both at mRNA and protein levels. These data suggested a potential correlation between tERG and IGF-1R.

**Figure 8.** Evaluation of IGF-1R and IR basal expression in prostate cell lines. Relative mRNA expression levels (top) of *IGF-1R* and *IR* in prostate cancer cell lines. The RWPE-1 cell line was used as a calibrator ($2^{ΔΔCt} = 1$). Protein expression levels of receptors in prostate cells (bottom). The blots are representative of two independent experiments.
1.2 Functional evaluation of tERG/IGF-1R correlation in PCa

For a comprehensive evaluation of the putative relationship between T2E and IGF-1R, different approaches were followed and are shown in figure 9. First, a transient silencing with siRNA directed against ERG was performed in VCaP cells. Interestingly, upon 96 and 120 hours of ERG silencing a down-modulation of IGF-1R was evidenced at protein levels compared to untreated cells or scrambled (Figure 9A). Second, IGF-1R expression was evaluated in RWPE-1 cells stably transfected for tERG overexpression (RWPE-1_tERG). The analysis pointed out that over-expression of ERG corresponded to a higher expression of IGF-1R both at mRNA and protein levels (Figure 9B). Third, as T2E fusion gene is regulated by androgens, IGF-1R expression was investigated in VCaP cells upon abiraterone acetate stimulation. Abiraterone acetate is a second-generation anti-androgen drug that blocks the synthesis of androgens. VCaP cells were treated for 72, 96 and 120h with two concentrations of abiraterone acetate and western blotting analysis showed that together with a strong ERG down-regulation, IGF-1R levels decreased upon 10 µM treatment in VCaP cells (Figure 9C). To better address the role of tERG in IGF-1R modulation, putative transcriptional regulation of ERG on IGF-1R promoter was investigated. An anti-ERG chromatin immunoprecipitation (ChIP) assay was performed in VCaP and PC-3 cells which express ERG at high or low levels, respectively, as well as in RWPE-1_tERG and RWPE-1 empty vector transfected cells. As shown in figure 10, ChIP analysis indicated that ERG binds the IGF-1R gene promoter, and the amount of binding was higher in cells with tERG expression. Considering the increase at mRNA levels in VCaP and RWPE-1_tERG compared to PC-3 or empty vector-transfected cells (Figures 8 and 9), the results indicate that ERG directly regulates IGF-1R transcription in PCa and that the increased amount of ERG due to fusion gene causes the higher IGF-1R expression in T2E-positive cells.
**Figure 9.** tERG-dependent IGF-1R induction in prostate cancer cells. A, siRNA knockdown of ERG (siERG) in VCaP induces a decrease in IGF-1R levels compared with non-treated control (NT) or non-targeting siRNA (SCR) controls. B, IGF-1R is over-expressed in RWPE-1 cells transfected with tERG compared with controls both at mRNA and protein level. Absolute *IGF-1R* mRNA quantification was assessed in RWPE-1 cells over-expressing t-ERG or empty vector-transfected cells (left). The blots are representative of two independent experiments (right). C, Abiraterone acetate treatment induces down-regulation of ERG in VCaP cells and IGF-1R. Cells were treated with abiraterone (3 and 10 µM) for the indicated time points. Representative blots are shown. GAPDH was used for normalization.
Figure 10. ERG acts as a stimulatory transcription factor in PCa cells. ChIP assay (top) was performed on VCaP and PC-3 prostate cancer cells, as well as on tERG- or empty vector-transfected RWPE-1 cells. ERG was precipitated with an anti-ERG-1/2/3 antibody. The results were obtained by quantitative RT-PCR. The data represent the recovery of each DNA fragment relative to the total input DNA. A representative experiment is shown. Cartoon (bottom) of the androgen/T2E/IGF-1R axis described in this study.
1.3 Efficacy of anti-IGF-1R agents in prostate cancer cells

As described in introduction section, several agents have been developed to specifically target IGF-1R on the basis of its importance in sustaining cell growth of different tumors. In PCa, several preclinical and clinical studies testing the effects of these agents have been performed and evidenced a limited efficacy. In this field, relevance of T2E in modulating the response to this type of treatment has been never considered. PCa cell lines were exposed for 72 hours to increasing concentrations of CP-751,871 or AVE1642, two anti-IGF-1R HAbs, as well as NVP-AEW541, a selective IGF-1R tyrosine kinase inhibitor (TKI). As shown in figure 11, an overall resistance to these agents was evidenced in the analyzed panel of cells with the exception of VCaP cells showing a remarkably high sensitivity to all anti-IGF-1R agents.
Figure 11. Sensitivity to IGF-1R inhibition in PCa cells. Cell growth was assessed using an MTT assay after a 72-h exposure to CP-751,871 or AVE1642, two anti-IGF-1R-HAbs, and NVP-AEW541, an anti-IGF-1R tyrosine kinase inhibitor (TKI) in prostate cell lines. The results are displayed as the percentage of survival relative to controls. Points, mean of two independent experiments; bars, SE.

To address the role of T2E/IGF-1R axis in influencing sensitivity to anti-IGF-1R agents, VCaP cells were transiently transfected with anti-ERG siRNA for 48 hours and treated with two concentrations of CP-751,871 HAb or NVP-AEW541 TKI corresponding to the doses conferring 20 and 50% of cell growth inhibition. Cell count showed that ERG expression significantly influenced efficacy of anti-IGF-1R agents as its silencing reverted cell sensitivity toward CP-751,871 or NVP-AEW541 in VCaP cells (Figure 12).
Figure 12. tERG overexpression increases sensitivity to anti-IGF-1R agents. ERG silencing was achieved in VCaP cells after a 48-h transfection of siERG (100 nM) or scrambled control siRNA (100 nM); GAPDH was used as a loading control. Cell survival is shown as the percentage of growth respect to untreated control. The data represent the mean values of two independent experiments, and the bars represent the SE.
1.4 Combinatory treatment of anti-IGF-1R therapy and anti-androgens

In PCa, anti-IGF-1R inhibitors effects have been investigated in combination with other drugs such as mitoxantrone (NCT00683475) or docetaxel [363]. TMPRSS2-ERG is driven by androgens and, consequently, all its down-stream effects can be putatively affected by androgens deregulation. In this landscape, sensitivity to abiraterone acetate was first assessed in a small panel of PCa cells. Accordingly to hormone response features, VCaP cells displayed a sensitivity to abiraterone acetate, with an calculated IC$_{50}$ of 20µM (Figure 13), while PC-3 and DU-145 cells were substantially resistant to the treatment. In VCaP cells, the doses of 3 and 30µM, conferring 20 and 50% of cell growth inhibition, respectively, were chosen for further experiments.

Figure 13. Sensitivity to Abiraterone acetate in PCa cells. Cell growth was assessed using an MTT assay after a 72-h exposure to Abiraterone acetate at the indicated doses. Results are displayed as the percentage of survival relative to controls. Points, mean of two independent experiments; bars, SE.
Response to abiraterone acetate in VCaP cells upon ERG silencing was investigated. As already demonstrated in literature, patients harboring T2E better respond to hormone treatment. Accordingly, ERG deprivation in VCaP cells induced a significant decrease in sensitivity to abiraterone stimulation compared to non treated or scrambled controls (Figure 14).

![Figure 14](image)

**Figure 14.** Reversion of sensitivity to abiraterone acetate by ERG knockdown. ERG was silenced in VCaP cells with siERG (100 nM) or scrambled control siRNA (100 nM); GAPDH was used as a loading control. Cells were treated with abiraterone acetate for 72 h at the indicated doses, and the survival percentage with respect to untreated control is shown. The data represent the mean values of two independent experiments, and the bars represent SE.

Interestingly, simultaneous administration of CP-751,871 and Abiraterone acetate but not cabazitaxel, a microtubule inhibitor recently introduced in PCa treatment, induced synergistic anti-proliferative effects in VCaP cells. In figure 15, individual doses of CP-751,871, abiraterone acetate or cabazitaxel to achieve 90% growth inhibition (blue line; ED90), 75% growth inhibition (green line; ED75) and 50% (red line; ED50) growth inhibition are plotted on the x- and y- axes.
Drug-drug interaction was classified as synergistic when CI was lower than 0.90, as additive when $0.90 \leq CI \leq 1.10$, or as subadditive when CI was higher than 1.10.

**Figure 15.** The combination of an IGF-1R inhibitor with Abiraterone acetate (top), but not cabazitaxel (bottom), results in synergistic effects in TMPRSS2-ERG-positive cells. The CI values representing ED90 are reported.
2. ETS rearrangements and IGF-1R expression in Ewing sarcoma

Considering the results obtained in PCa regarding ERG capabilities to regulate IGF-1R gene expression, activity of EWS-FLI1 on IGF-1R was investigated. EWS-FLI1 is the hallmark of ES and several studies have been performed in the past years in order to evidence target genes that could be de-regulated by EWS-FLI1. EWS-FLI1 has been already demonstrated to influence transcriptional regulation of some IGF system components, including IGF-1 and IGFBP-3, but not IGF-1R [92].

To address the EWS-FLI1 activity on IGF-1R, ES cell line TC-71 was transiently transfected with two concentrations of anti-FLI1 siRNA and protein expression of FLI1 and IGF-1R was evaluated by western blotting. As shown in figure 16, knock-down of EWS-FLI1 induced a down-regulation of IGF-1R protein highlighting a new interesting mechanism in ES biology. Moreover, an anti-FLI1 chromatin immunoprecipitation (ChIP) assay was performed in TC-71, 6647 and LAP-35 ES cell lines. TC-71 represents a model of EWS-FLI1 type 1 chimera while 6647 and LAP-35 cells display EWS-FLI1 type 2 hybrid. ChIP analysis evidenced the recruitment of FLI1 to IGF-1R promoter in all of the cellular models (Figure 17).

Figure 16. EWS-FLI1 knockdown in TC-71 cells induces a decrease in IGF-1R levels compared with non-treated control or scrambled control.
3. Effects of Trabectedin (ET-743, Yondelis\textsuperscript{TM}) on ETS fusion genes binding to IGF-1R promoter

Recently, capability of DNA binding agents including trabectedin has been demonstrated to modulate activity of transcription factors, introducing a certain specificity and a new level of complexity in the action of conventional agents. Such effects have been already described in myxoid liposarcoma and ES where trabectedin interferes with the activity of FUS-CHOP and EWS-FLI1, respectively, justifying the elevated sensitivity to trabectedin of tumors harboring fusion genes. Trabectedin is a tetrahydroisoquinoline molecule that binds to the N2 of guanine in the minor groove, causing DNA damage and affecting transcription regulation in a promoter- and gene-specific manner. In this study, the effects of trabectedin on IGF-1R were investigated.
based on the evidence that ES cell variants resistant to trabectedin displayed an up-regulation of IGF-1R and IRS-1 gene expression compared to parental cells [364].

### 3.1 Evaluation of EWS-FLI1 binding to IGF-1R promoter upon stimulation with trabectedin

Trabectedin is one of the very few novel drugs recently proposed for the treatment of sarcoma patients. However, the activity observed in ES was quite modest in the clinical setting. Identification of mechanisms that could improve the efficacy of trabectedin in ES represents the main goal to optimize the use of this drug. The study was performed in TC-71 and 6647 cell lines as representative models of type 1 and type 2 EWS-FLI1 chimera, respectively. Anti-FLI1 ChIP assay and Real Time-PCR were performed to monitor the binding of EWS-FLI1 to a conventional target gene, TGFβR2, as well as IGF-1R promoter upon trabectedin or doxorubicin (DXR) stimulation. Cell lines were treated with different concentrations of trabectedin or DXR for 1 hour, IC₅₀ doses were calculated and used for the experiments. Treatment with Trabectedin (2.5nM and 10nM, respectively) or DXR (1μM and 2μM, respectively) induced a significant reduction of EWS-FLI1 binding to TGFβR2 promoter (Figure 18). Conversely, trabectedin but not DXR caused an increased recruitment of EWS-FLI1 on IGF-1R promoter. As shown in figure 19, occupancy of EWS-FLI1 to IGF-1R promoter was dose- and time-dependent. Interestingly, trabectedin-induced up-regulation of the IGF-1R was also confirmed at protein level and the analysis pointed out that IGF-1R increase was maintained up to 48 hours (Figure 19).
Figure 18. Trabectedin induces detachment of EWS-FLI1 from specific promoters. ChIP assay results were obtained by quantitative RT-PCR and data are reported as fold enrichment over the control. * p<0.05; ** p<0.001, Student’s t test.
Figure 19. Trabectedin induces recruitment of EWS-FLI1 on IGF-1R promoter in ES cells. A, ChIP assay results in TC-71 and 6647 ES cells treated for 1h with trabectedin or DXR. Data represent recovery of each DNA fragment relative to total input DNA, respect to control. * p<0.05; Student’s t test. B, Time-course of EWS-FLI1 association with IGF-1R promoter evaluated by quantitative RT-PCR in TC-71 cells. Bars represent SE. * p<0.05; ** p<0.001, Student’s t test. C, Up-regulation of IGF-1R at protein level by western blotting after exposure of TC-71 cells to trabectedin (0.5-1 nM) up to 48h. GAPDH was used as loading control.
3.2 Evaluation of tERG binding to IGF-1R promoter upon stimulation with trabectedin

Evidences regarding efficacy of trabectedin as single agent in PCa are limited to one phase II clinical trial carried out in metastatic castration-resistant patients. In this population of PCa patients, few therapeutic options are available and the strategies currently in use confer transient benefits evidencing that additional agents are needed. In the study conducted by Michaelson et al., Trabectedin was administered following two schedules but the results evidenced modest activity of this agent with a 13% of patients experiencing a PSA decrease ≥50% [365]. Identification of patients with specific molecular features who could benefit of trabectedin treatment could improve use of this agent in clinic.

In this study, VCaP cells were treated for 1h with two doses of trabectedin (1 and 3nM) and ChIP assay with anti-ERG antibody was performed. Doses were decided based on cell count upon 1h treatment with a range of concentrations of trabectedin. ChIP assay showed that trabectedin is able to displace ERG binding to PIM-1 promoter, a conventional ERG target gene. Conversely to the results obtained in ES, trabectedin also induced ERG detachment from IGF-1R promoter, evidencing a cellular-dependent activity of trabectedin on IGF-1R promoter. Results of ChIP assay are shown in figure 20.

**Figure 20.** ChIP assay results displaying that Trabectedin reduces ERG binding to PIM-1 as well as IGF-1R promoter in VCaP cell line after 1 hour of treatment. Results were obtained by quantitative RT-PCR and data are reported as fold enrichment over the control. **p<0.001, Student’s t test.**
3.3 Therapeutic implications of trabectedin gene-specific effects

Considering the increased IGF-1R expression in ES cells upon treatment with trabectedin and the importance of IGF-1R in sustaining ES cells growth, TC-71, 6647 and LAP-35 cell lines were treated with a combination of trabectedin and the anti-IGF1R/IR dual inhibitor OSI-906. OSI-906 was chosen because it has been already reported that high levels of IR can overcome IGF-1R blockade in ES cells. Interestingly, the combined treatment gave synergistic effects in ES cell lines. On the contrary, combination of trabectedin with OSI-906 gave subadditive effects in VCaP PCa cells harboring T2E rearrangement, according to inhibitory effects of trabectedin on IGF-1R promoter occupancy (Table 3).

These results suggest that a combination of trabectedin and anti-IGF-1R inhibitor represents a new potential therapeutic option for ES but not for PCa patients.

Table 3. Efficacy of Trabectedin and OSI-906 as single agents or in combination in ES and PCa cell lines.

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<th>Trabectedin (nM) IC(_{50}) ± SE</th>
<th>OSI-906 (µM) IC(_{50}) ± SE</th>
<th>Dose ratio</th>
<th>Drug combination CI ± SE</th>
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<td>0.17 ± 0.07</td>
<td>1:1000</td>
<td>0.734±0.08</td>
<td>synergistic</td>
</tr>
<tr>
<td>VCaP</td>
<td>1.96 ± 0.12</td>
<td>0.18 ± 0.5</td>
<td>1:100</td>
<td>1.43 ± 0.05</td>
<td>subadditive</td>
</tr>
</tbody>
</table>
4. Prognostic relevance of IGF system and assessment of TMPRSS2-ERG/IGF-1R axis in PCa patients

4.1 Gene expression profile of IGF system in primary PCa

Clinical role of IGF system was previously investigated in ES in order to identify patients with distinct outcome and putatively different treatment protocols. Results showed that transition to frank malignancy is associated with a reduction of IGF system activity [282]. Prognosis value of IGF system and mainly of IGF-1R in PCa still remains controversial. Noteworthy, T2E has been already reported to identify patients with different clinico-pathological characteristics and different prognosis. Gene expression of IGF-1R, IR, IGF-1, IGF-2 and IGF-BP3 was evaluated in a retrospective series of 270 primary prostate tumors by Real-Time PCR (Table 2). While no IGF-2 expression was detected in samples, differential expression of IGF-1R, IGF-1, IGFBP-3 and IR was compared to normal prostate tissues following the $2^{-\Delta\Delta Ct}$ method. As shown in figure 21, no differential expression with respect to normal tissue was noticed for IGF-1R (median=1,04; range=0,07-5,12), while a variable expression was evidenced for IGF-1 (median=0,61; range=0,01-50,12) and IR (median=0,58; range=0,01-471,75). IGFBP-3 was found to be substantially down-regulated (median=0,52; range=0,05-2,96).

![Figure 21. IGF system expression profile in prostate cancer patients.](image-url)
Expression of the genes was classified in “high” and “low” expression depending on if obtained RQ values were above or below the first quartile respectively. The association of IGF-1R, IR, IGF-1 and IGF-BP3 expression with clinico-pathological characteristics and prognosis was analyzed. Fisher’s test pointed out an association between IGF-1R and T2E expression in clinical samples (p=0.008). Particularly, patients harboring the fusion gene showed higher IGF-1R mRNA levels, in keeping with the increased binding of ERG to the IGF-1R promoter observed in the experimental models. No other statistically significant correlations were found.

Kaplan-Meier and Log-rank tests were applied in order to evaluate the prognostic role of IGF system components for both biochemical progression free survival (BPFS) and clinical progression free survival (PFS) (Table 5). The analysis pointed out a statistically significant association between high expression of IGF-1 and good BPFS or PFS while a borderline association was evidenced between high expression of IGF-1R and better BPFS. IGF-1 expression but not IGF-1R expression was significant in the Cox proportional hazard multivariable analysis (hazard ratio (HR): 0.62, IC 95% [0.41-0.94], p=0.026) respect to BPFS. Since IGF-1R was found associated with the presence of the T2E translocation both in cell lines and in patients, the series was divided depending on the status of the fusion gene thus identifying two cohorts of patients: T2E-positive or –negative, respectively. In the two groups, IGF-1R, IR, IGF-1 and IGF-BP3 gene expression was classified in “high”, if the RQ value was above the first quartile, and “low” expression, if RQ value was below the first quartile. Then, association with clinico-pathological features and prognosis were assessed (Tables 6 and 7). No association with clinico-pathological characteristics was found in any of the two groups. Interestingly, Kaplan-Meier analysis evidenced that prognostic value of IGF-1R was statistically stronger in T2E-negative patients (p-value = 0.016) while it was not associated with survival in T2E-positive subgroup (p-value > 0.5). More in detail, the analysis pointed out that in patients negative for the fusion gene, a lower expression of IGF-1R confers a worse prognosis considering BPFS. This results further underlies the importance of T2E in establishing subgroups of patients with different prognosis. IGF-1 was found associated with BPFS and PFS in all of the subgroups (Figure 22).

Accordingly to multivariate analysis, nor IGF-1 or IGF-1R represent independent variables influencing prognosis in T2E-positive or –negative subgroups.
Prognostic value of IGF-1R and IGF-1 transcripts on BPFS of primary PCa patients undergone radical prostatectomy without previous treatment. The total series was divided into two groups depending if T2E was expressed or not. Comparison of survival curves was performed by the log rank test. Time scale refers to months from diagnosis. Thick lines indicate high expressing patients. BPFS, biochemical progression free survival.

4.2 Protein expression of IGF-1R, IR and ERG in primary PCa tissues and association with prognosis

Protein expression of IGF-1R, IR and ERG was analyzed in 243 cases from the same series (Table 2). Expression of IGF-1R in primary PCa tissues was not detectable in 21/243 cases and negative in 12/222 of the samples (5%). Among the positive cases, 85% was classified as high-expressors (165/210). IR was found not detectable in 17/243 cases and negative in 51/226 of the samples (22.5%). Among the positive cases, 34%
was classified as high-expressors (76/226). ERG expression was not detectable in 24/243 and negative in 85/219 cases (39%) and 85% of them was classified as high-expressors. Interestingly, a statistically significant correlation was found between ERG protein levels and classical T2E evaluation reported in Material and Methods section (p < 0.0001, Fisher’s test). In addition, IGF-1R protein levels were significantly associated with mRNA levels (p=0.047, Fisher’s test). IGF-1R was associated with ERG protein levels (p<0.0001; Fisher’s test), further verifying the association between IGF-1R and T2E (Figure 23), but no other association was evidenced. Kaplan-Meier analysis was performed in the whole series as well as in ERG-positive and negative groups but no association with BPFS or PFS was found at protein level (Tables 8, 9 and 10).

**Figure 23.** Representative expression of ERG (top) and IGF-1R (bottom) in prostate cancer tissue array samples by immunohistochemistry (magnification, x40). The cases were classified as ‘high-expressors’ when medium or high positivity was present and ‘low-expressors’ when no staining or low positivity was observed.
5. TMPRSS2-ERG and CD99 molecule

5.1 Preliminary data

Preliminary data, previously obtained in the laboratory where this work has been conducted, evidenced that CD99 acts as putative oncosuppressor in PCa. PC-3 cell line was stably transfected for over-expression of CD99wt, as shown in figure 24. Transfectant cells displayed an attenuate phenotype as demonstrated by a reduced anchorage-independent growth (soft-agar assay) and a decreased migration capability when compared to non treated or empty vector transfectant cell lines.

![Western Blot](image.png)

**Figure 24.** CD99 acts as an oncosuppressor in PCa cells. PC-3 cells were transfected for CD99wt and its over-expression was evaluated by western blotting (top). Migratory features of PC-3 parental and transfected cells (bottom right). Growth in soft-agar of PC-3 and clones (bottom left) [347].
In vivo, PC-3 cells over-expressing CD99 showed a minor tumor incidence and a decreased number of extrapulmonary metastases in mice as well as a higher tumor latency with respect to parental cells [347] (Figure 25).

Figure 25. CD99 inhibits PCa cell metastatization in mice [347].

Clinical relevance of this data was confirmed by CD99 immunohistochemical analysis performed in prostate tissues spanning from hyperplasias to primitive tumor and metastases. In patients, CD99 expression was higher in benign lesion (70% positivity) when compared to primitive (12.5% positivity) or metastases (30% positivity) tissues (Figure 26).
Figure 26. CD99 evaluation by immunohistochemistry in patients. a) Benign prostatic hyperplasia b) Prostate adenocarcinoma grade II c) Prostate adenocarcinoma grade III d) Bone metastasis from prostate cancer.

As described in introduction section, the primary site for PCa metastasis is bone and different factors take part in establishing this process including “homing” events, capability to stimulate osteoblastic lineage and osteomimicry aptitude of PCa cells. A preliminary clue indicating a putative role of CD99 in PCa bone metastasis was obtained in this laboratory demonstrating particularly that PC-3 cells over-expressing CD99 showed an overall down-regulation of genes involved in osteoblastic differentiation. The results were obtained by GeneCARDs analysis showing that PC-3 cells over-expressing CD99 showed a lower expression of genes involved in ossification, mineralization, bone development, cell-cell adhesion, cell-matrix adhesion, matrix proteases and proteases inhibitors and transcription factors. Accordingly, expression of proteins involved in osteoblastic differentiation were found higher in primitive or metastases PCa lesions compared to benign lesions. Representative immunohistochemistry images of the analyzed biomarkers including osteopontin, bone sialoprotein, osteonectin and osteocalcin are shown in figure 27. Percentages of positivity in the analyzed series is reported in table 4.
Table 4. Percentages of cases positive for osteoblastic differentiation markers.

<table>
<thead>
<tr>
<th>% of positive cases</th>
<th>Osteopontin</th>
<th>Bone sialoprotein</th>
<th>Osteonectin</th>
<th>Osteocalcin</th>
</tr>
</thead>
<tbody>
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<td>Hyperplasias</td>
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<td>30</td>
<td>0</td>
<td>50</td>
</tr>
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<td>Primitive</td>
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<td>80</td>
<td>77</td>
</tr>
<tr>
<td>Metastasis</td>
<td>78</td>
<td>50</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure 27. Immunohistochemical analysis of osteoblastic differentiation markers in hyperplasias, primitive tumor and metastasis.
5.2 Analysis of CD99 association with survival in primitive PCa samples

In this section of the study, CD99 expression levels were analyzed in a large cohort of primitive PCa specimens both at gene and protein levels (Table 2). Subsequently, correlation between CD99 expression and clinico-pathological parameters as well as BPFS or PFS was investigated. Studies focused on CD99 prognosis value are limited and particularly none of them was related to PCa. Gene expression of CD99 was evaluated in a retrospective series of 270 primary prostate tumors by Real-Time PCR. Comparison with normal prostate tissues revealed a slight lower expression of CD99 in tumor compared to healthy tissues (median value=0.83; range=0.2-2.28) (Figure 28).

![Figure 28. CD99 expression profile in prostate cancer patients.](image)

Expression of CD99 was classified in “high” and “low” expression depending on if obtained RQ value was above or below the first quartile, respectively. The association with clinico-pathological characteristics and prognosis was analyzed. Fisher’s test pointed out an association, marginal at best, between CD99 and T2E expression (p=0.054). Particularly, patients harboring the fusion gene showed higher CD99 mRNA levels. In addition, CD99 loss was found associated with positivity of margins (p=0.034, Fisher’s test), index of a more aggressive disease and in accordance to preliminary data indicating a role of CD99 in metastatic process.
Kaplan-Meier and Log-rank tests were applied in order to evaluate the prognosis role of CD99 for both BPFS and PFS (Table 5). The analysis pointed out that CD99 is not statistically associated with survival but a trend of the curves indicated that higher expression of CD99 confers a slight better outcome accordingly to the indicated oncosuppressive value of CD99 in PCa (Figure 29).

![BPFS graph](image)

**Figure 29.** CD99 expression and association with BPFS or PFS in PCa. Time scale refers to months from diagnosis. Thick lines indicate high expressing patients. BPFS, biochemical progression free survival.

Since CD99 was found associated with T2E translocation, the series was divided depending on the status of the fusion gene and CD99 gene expression was classified in “high”, if the RQ value was above the first quartile, and “low” expression, if RQ value was below the first quartile in the two groups. Fisher's test pointed out no association with clinico-pathological features or BPFS and PFS in any of the two groups.
At protein level, CD99 was analyzed by Tissue Microarray in the retrospective series of 243 cases. Expression of CD99 in primary PCa tissues was detected in 23/243 samples. Among the positive cases, 42% was classified as high-expressors (94/220). Among clinico-pathological parameters, CD99 was associated with ERG protein levels (p < 0.01 Fisher’s test), as T2E-positive cases correlated with higher CD99 expression. Kaplan-Meier analysis was performed in the whole series as well as in ERG-positive and-negative groups but no association with BPFS or PFS was found at protein level.

5.3 Analysis of CD99 expression in PCa cell lines

CD99 gene and protein levels were investigated in a panel of PCa cell lines including malignant VCaP, DU-145, PC-3, LNCaP, 22RV1 and the non-malignant RWPE-1 cells. As shown in figure 30, CD99 was found similarly expressed between PCa cells and RWPE-1 cells with exception of LNCaP and 22RV1 cells which particularly displayed a consistent down-modulation of CD99 at mRNA level. No remarkable difference was noticed in VCaP cells at basal level compared to other cell lines.
5.4 In vitro analysis of ETS rearrangements/CD99 correlation

Considering data obtained in patients, VCaP cells were transiently transfected with ERG siRNA and CD99 expression was investigated to better elucidate a putative correlation between fusion gene and CD99. Interestingly, 48 hours silencing of ERG induced a down-regulation of CD99 at protein level but no modulation of \( CD99 \) transcript was found (Figure 31). These data better reflect results obtained in patients highlighting a stronger correlation between CD99 and ERG at protein level more than at mRNA level. ETS rearrangements appear to be able to influence CD99 expression but no through a direct transcriptional regulation. Accordingly, anti-ERG ChIP analysis was performed in VCaP and PC-3 cells as well as in RWPE-1_ERG or empty vector transfected cells.

**Figure 30.** Real Time-PCR (top) and western blotting (bottom) analysis of CD99 in prostate cancer cells.
and qRT-PCR was perform to evaluate recruitment to CD99 promoter. As shown in figure 32, a fraction of ERG was actually found to bind CD99 gene promoter but the amount of binding was not different in VCaP and RWPE-1_tERG models compared to PC-3 or RWPE-1 empty vector transfected cells.

**Figure 31.** ERG influences CD99 at protein level but not at mRNA level. siRNA knockdown of ERG (siERG) in VCaP was assessed by western blotting analysis. mRNA and protein expression of CD99 was evaluated by Real Time-PCR (bottom left) and western blotting (bottom right), respectively. A representative experiment is shown.
Figure 3. ChIP assay was performed on VCaP and PC-3 prostate cancer cells, as well as on tERG- or empty vector-transfected RWPE-1 cells. ERG was precipitated with an anti-ERG-1/2/3 antibody. The results were obtained by quantitative RT-PCR. The data represent the recovery of each DNA fragment relative to the total input DNA. A representative experiment is shown.

As a further confirmation of the marginal or indirect role of ERG in regulating CD99 at transcriptional levels, trabectedin treatment in VCaP or PC-3 cell lines did not affect ERG binding to CD99 promoter (Figure 33). Conversely, previous data in ES showed that EWS-FLI1 actually is able to bind CD99 promotor thus regulating its expression [94]. Accordingly, trabectedin treatment in TC-71 and 6647 ES cell lines induced a displacement of the chimera from CD99 promoter (Figure 34).
Figure 33. Trabectedin reduces ERG binding to PIM-1 but not CD99 promoter in VCaP cell line after 1 hour of treatment. Results were obtained by quantitative RT-PCR and data are reported as fold enrichment over the control. ** p<0.001, Student’s t test.

Figure 34. Trabectedin induces detachment of EWS-FLI1 from CD99 promoter. ChIP assay results were obtained by quantitative RT-PCR and data are reported as fold enrichment over the control. * p<0.05; ** p<0.001, Student’s t test.
Table 5. BPFS and clinical PFS log rank and Cox regression tests in primary PCa analyzed by qRT-PCR.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No.</th>
<th>Total cases</th>
<th>Biochemical Progression</th>
<th>Cliniopathological Progression</th>
<th>Clinical Progression</th>
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<td></td>
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<td></td>
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<tr>
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<td>15</td>
<td>15 (64,2)</td>
<td>0,245</td>
<td></td>
<td>0,379</td>
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<tr>
<td>56-65</td>
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<td>43 (25,7)</td>
<td>0,379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66-75</td>
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<td>0,379</td>
<td></td>
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<td>&gt; 75</td>
<td>36</td>
<td>18 (48,6)</td>
<td>0,379</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0,015</td>
</tr>
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<td>129</td>
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<td></td>
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<td></td>
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<td></td>
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<tr>
<td>cT2b or less</td>
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<td>108 (42,3)</td>
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<td>1</td>
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<td>16 (14,5)</td>
<td>&lt;0,0001</td>
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<td></td>
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</tr>
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<td>43 (57,4)</td>
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<td>84 (20,4)</td>
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<td>TMPRSS2/ERG</td>
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<td>IGF-1R</td>
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<tr>
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<td>88 (43,4)</td>
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</table>
Table 6. BPFS and clinical PFS log rank and Cox regression tests in patients with PCa and TMPRSS2-ERG fusion gene expression analyzed by qRT-PCR.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Biochemical Progression</th>
<th>Clinical Progression</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>No. Pts</td>
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<tr>
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<td></td>
</tr>
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<td>56-65</td>
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<td>&gt; 75</td>
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<td>Gleason-sp:</td>
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<tr>
<td>2-6</td>
<td>72</td>
<td>20 (64,3)</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>43 (22,2)</td>
</tr>
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<tr>
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<td>0,889</td>
</tr>
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<td>Low</td>
<td>44</td>
<td>17 (60)</td>
</tr>
<tr>
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<td>134</td>
<td>58 (43,1)</td>
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<tr>
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<tr>
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<td>57 (42)</td>
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<tr>
<td>IGF-1</td>
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<tr>
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<tr>
<td>High</td>
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Table 7. BPFS and clinical PFS log rank and Cox regression tests in patients with PCa and no TMPRSS2-ERG fusion gene expression analyzed by qRT-PCR.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Biochemical Progression</th>
<th>Clinical Progression</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. Pts</td>
<td>No. Events (% BPFS)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 55</td>
<td>5</td>
<td>1 (80)</td>
</tr>
<tr>
<td>56-65</td>
<td>22</td>
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<tr>
<td>66-75</td>
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<td>&gt; 75</td>
<td>9</td>
<td>6 (33,3)</td>
</tr>
<tr>
<td>Gleason-sp:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-6</td>
<td>37</td>
<td>15 (43,1)</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
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<td>Greater than 20</td>
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<td>11 (15,4)</td>
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<tr>
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<tr>
<td>cT2b or less</td>
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<tr>
<td>cT3a or greater</td>
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<td>10 (0)</td>
</tr>
<tr>
<td>pT:</td>
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<td></td>
</tr>
<tr>
<td>pT2 or less</td>
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<tr>
<td>pT3 or greater</td>
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<td>28 (26)</td>
</tr>
<tr>
<td>pN:</td>
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<td></td>
</tr>
<tr>
<td>pN0</td>
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</tr>
<tr>
<td>pN1 or greater</td>
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<tr>
<td>Margins:</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>Positive</td>
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<td>IGF-1R</td>
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<tr>
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<td>23</td>
<td>15 (34,8)</td>
</tr>
<tr>
<td>High</td>
<td>69</td>
<td>34 (35,3)</td>
</tr>
<tr>
<td>CD99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>23</td>
<td>15 (16,9)</td>
</tr>
<tr>
<td>High</td>
<td>69</td>
<td>34 (40,2)</td>
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Table 8. BPFS and clinical PFS log rank and Cox regression tests in primary PCa analyzed by IHC.

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<th>Parameter</th>
<th>Biochemical Progression</th>
<th>Clinical Progression</th>
</tr>
</thead>
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<td>Univariate p Value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>Univariate p Value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>No. Pts</td>
<td>No. Events (% BPFS)</td>
<td>No. Events (% PFS)</td>
</tr>
<tr>
<td>No. Events</td>
<td>Univariate p Value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Gleason-sp:</td>
<td>2-6</td>
<td>87 26 (57,7)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>123 60 (29,5)</td>
</tr>
<tr>
<td></td>
<td>Greater than 7</td>
<td>29 23 (11,5)</td>
</tr>
<tr>
<td>PSA (ng/ml):</td>
<td>10 or less</td>
<td>132 49 (45,5)</td>
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<tr>
<td></td>
<td>10-20</td>
<td>69 34 (40,5)</td>
</tr>
<tr>
<td></td>
<td>Greater than 20</td>
<td>36 25 (26,4)</td>
</tr>
<tr>
<td>cT:</td>
<td>cT2b or less</td>
<td>219 95 (41,4)</td>
</tr>
<tr>
<td></td>
<td>cT3a or greater</td>
<td>19 14 (17,6)</td>
</tr>
<tr>
<td>pT:</td>
<td>pT2 or less</td>
<td>115 37 (55,9)</td>
</tr>
<tr>
<td></td>
<td>pT3 or greater</td>
<td>124 72 (23,4)</td>
</tr>
<tr>
<td>pN:</td>
<td>pN0</td>
<td>209 91 (42,2)</td>
</tr>
<tr>
<td></td>
<td>pN1 or greater</td>
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</tr>
<tr>
<td>Margins:</td>
<td>Negative</td>
<td>116 33 (54,8)</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>123 76 (21,5)</td>
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<td>ERG intensity:</td>
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<td></td>
<td>Positive</td>
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<tr>
<td></td>
<td>High expressors</td>
<td>165 79 (34,6)</td>
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<td>IR intensity:</td>
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<tr>
<td></td>
<td>High expressors</td>
<td>74 37 (32,8)</td>
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<tr>
<td>CD99 intensity:</td>
<td>Low expressors</td>
<td>123 58 (30,9)</td>
</tr>
<tr>
<td></td>
<td>High expressors</td>
<td>93 44 (43,1)</td>
</tr>
</tbody>
</table>
Table 9. BPFS and clinical PFS log rank and Cox regression tests in patients with PCa and TMPRSS2-ERG positive analyzed by IHC.

| ERG Positive | Biochemical Progression | | Clinical Progression | |
|--------------|-------------------------|--------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|
| Parameter    | No. Pts | No. Events (% BPFS) | Univariate p Value | HR (95% CI) | Multivariate p Value | No. Events (% PFS) | Univariate p Value | HR (95% CI) | Multivariate p Value |
| Gleason-sp:  |          |                       |                      |              |                        |                      |                      |              |                        |
| 2-6          | 43       | 13 (59,6)             | <0,0001              | 1            | 0,003                  | 0,001                | 5 (78,6)             | 0,001        | Not significant       |
| 7            | 61       | 32 (30,9)             | 1                     | 5,98 (2,15-16,6) | 24 (45,8)             | 0,001                | 1 (87,5)             | 0,001        | Not significant       |
| Greater than 7 | 10      | 8 (20)                | >0,0001               | 0,16 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| PSA (ng/ml): |          |                       |                      |              |                        |                      |                      |              |                        |
| 10 or less   | 63       | 21 (56,8)             | <0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| 10-20        | 32       | 16 (38)               | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| Greater than 20 | 19      | 16 (0)                | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| cT:          |          |                       |                      |              |                        |                      |                      |              |                        |
| cT2b or less | 34       | 7 (74,1)              | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| cT3a or greater | 79    | 46 (28,7)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| pT:          |          |                       |                      |              |                        |                      |                      |              |                        |
| pT2 or less  | 56       | 18 (64,8)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| pT3 or greater | 58     | 35 (23,2)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| pN:          |          |                       |                      |              |                        |                      |                      |              |                        |
| pN0          | 92       | 40 (47,2)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| pN1 or greater | 6      | 6 (0)                 | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| Margins:     |          |                       |                      |              |                        |                      |                      |              |                        |
| Negative     | 59       | 17 (65)               | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| Positive     | 55       | 36 (12,9)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| IGF-1R intensity |       | 654                   | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| Low expressors | 10      | 4 (60)                | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| High expressors  | 104    | 49 (39,9)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| IR intensity  |          |                       |                      |              |                        |                      |                      |              |                        |
| Low expressors | 67      | 31 (45,1)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| High expressors | 47      | 22 (37,4)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| CD99 intensity |         | 175                   | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| Low expressors | 62      | 32 (33,3)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
| High expressors | 51      | 20 (50,9)             | >0,0000               | 0,11 (1,29-7,75) | 1 (87,5)             | 0,001                | 0,079                |            |                        |
Table 10. BPFS and clinical PFS log rank and Cox regression tests in patients with PCa and TMPRSS2-ERG negative analyzed by IHC.

| ERG Negative Parameter | Biochemical Progression | | | Clinical Progression | | |
|------------------------|-------------------------|-----------------|-----------------------|-------------------------|-------------------|
|                        | No. Events (BPFS) | Univariate p Value | HR (95% CI) | No. Events (PFS) | Univariate p Value | HR (95% CI) |
| **Gleason-sp:** | | | | | | |
| 2-6 | 32 | 11 (32,7) | 0,003 | Not significant | 6 (77,4) | 0,014 | Not significant |
| 7 | 53 | 27 (23,7) | | | 16 (66,3) | | |
| Greater than 7 | 16 | 12 (15,6) | | | 9 (0) | | |
| **PSA (ng/ml):** | | | | | | |
| 10 or less | 52 | 25 (16,9) | 0,810 | Not significant | 0 (0) | | 0,929 |
| 10-20 | 32 | 16 (43,6) | | | 9 (66,1) | | |
| Greater than 20 | 15 | 8 (44,4) | | | 4 (71,4) | | |
| **cT:** | | | | | | |
| cT2b or less | 93 | 44 (25,2) | 0,011 | Not significant | 10 (52,6) | 0,055 | Not significant |
| cT3a or greater | 8 | 6 (15) | | | 21 (55,9) | | |
| **pT:** | | | | | | |
| pT2 or less | 43 | 16 (30,2) | 0,013 | Not significant | 11 (72,2) | 0,226 | |
| pT3 or greater | 58 | 34 (18,7) | | | 20 (46,9) | | |
| **pN:** | | | | | | |
| pN0 | 93 | 45 (24,5) | < 0,0001 | Not significant | 29 (60,1) | 0,088 | |
| pN1 or greater | 4 | 4 (0) | 21,73 (6,36-71,42) | | 2 (0) | | |
| **Margins:** | | | | | | |
| Negative | 43 | 14 (23,1) | 0,003 | Not significant | 7 (78,6) | 0,015 | |
| Positive | 58 | 36 (31,3) | 2,71 (1,41-5,23) | | 24 (38,5) | 2,87 (1,23-6,71) | |
| **IGF-1R intensity** | | | | | | |
| Low expressors | 43 | 21 (25,7) | 0,786 | | 12 (68) | 0,45 | |
| High expressors | 58 | 29 (22,1) | | | 19 (55,8) | | |
| **IR intensity** | | | | | | |
| Low expressors | 74 | 33 (35,6) | 0,06 | | 20 (68,9) | 0,08 | |
| High expressors | 25 | 15 (27,9) | | | 10 (0) | | |
| **CD99 intensity** | | | | | | |
| Low expressors | 58 | 25 (29,2) | 0,078 | Not significant | 17 (66,7) | 0,64 | |
| High expressors | 41 | 24 (29,8) | | | 13 (44,5) | | |
Discussion

Several studies have pointed out the relevance of ETS family of transcription factors in pathogenesis and progression of several cancers. ETS proteins act as gene activators or repressors, regulating genes involved in proliferation, differentiation, apoptosis, metastasis, tissue remodeling, and angiogenesis. Expression of ETS genes has been found altered both in leukaemias and solid tumors and, particularly, an aberrant expression due to chromosomal translocations has been described as a common feature of different tumor types including ES, PCa and acute myeloid leukaemia. Presence of tumor-specific fusion genes represents a potential opportunity in clinic for identification of diagnosis or prognosis biomarkers and pharmacologic intervention to therapeutic benefit. More recently, interest in ETS-driven diseases has been further supported by finding that several chemotherapeutic drugs including cytarabine, doxorubicin [366] and etoposide [367] induce an ETS-attenuation gene expression signature while resulting in significant co-morbidities, including organ-toxicity, such as cardiomyopathy, or second malignancy due to shared molecular mechanisms between tumor and normal tissues [368, 369]. Taken together these evidences highlight the importance of transversal studies to unravel shared or distinct target genes, pathways and mechanisms of regulation in ETS-driven diseases. In this study, ES and PCa have been considered in order to identify common or distinctive mechanisms determined by ETS rearrangements. Particularly, impact of EWS-FLI1, the hallmark of ES, and TMPRSS2-ERG, in PCa, has been investigated on the IGF system and CD99 molecule. In ES, EWS-FLI1 has been demonstrated to regulate transcription of some IGF system components, including $IGF-1$ and $IGFBP-3$, acting as gene activator and repressor, respectively, as well as $CD99$ promoter. It has been recently demonstrated that ETS-positive prostate tumors display a greater under-expression of $IGFBP-3$ compared to ETS-negative [370]. The presented results demonstrate for the first time that $IGF-1R$ represents a common target of ETS rearrangements. ChIP analysis showed ERG and FLI1 binding to the $IGF-1R$ gene promoter, suggesting a direct transcriptional regulation of $IGF-1R$ by tERG in PCa or EWS-FLI1 in ES. Possibility to have common deregulated genes is in line with previous evidences demonstrating that tERG, EWS-ERG or FUS/ERG actually significantly up-regulate the transcript of more than 100 common genes, including PIM-1, in the NIH-3T3 cell line [174]. In PCa, a greater ERG recruitment to $IGF-1R$ promoter was found in VCaP cells compared to PC-3 or in RWPE-1_tERG compared to empty vector. In addition,
androgen deprivation induced by abiraterone acetate treatment in the androgen-responsive VCaP cells caused a decrease in the expression of ERG, as previously reported [371], but also an inhibition of IGF-1R confirming the presence of a TMPRSS2-ERG/IGF-1R androgen-regulated axis. The relationship between T2E and IGF-1R was also confirmed in radical prostatectomy specimens; patients expressing the fusion gene exhibited higher IGF-1R expression. TMPRSS2-ERG represents an early event in prostate cancer but its expression is maintained from primary tumor cells to metastatic cells [372]. Considering that RWPE-1 is a model of non-tumorigenic immortalized cells while VCaP cells are representative of advanced disease, the data indicate that TMPRSS2-ERG/IGF-1R axis may represent a constant mechanism along the different stages of the pathology. The IGF-1R gene has been identified as a molecular target for a number of stimulatory transcription factors and inhibitory proteins with important implications in cancer. Aberrant fusion product such as EWS-WT1, the genetic hallmarks of desmoplastic small round cell tumor, was found to act as transactivator for the IGF-1R gene, providing a selective growth advantage to tumor cells. Despite in contrast with previous evidences in literature [92], EWS-FLI1 was found to bind IGF-1R gene promoter indicating a transcriptional regulation. In addition, silencing of EWS-FLI1 was observed in parallel with down-regulation of IGF-1R suggesting that IGF-1R is indeed a target of EWS-FLI1. Identification of EWS-FLI1 targets represents a key aspect in the understanding of the molecular behavior of ES. It is widely recognized that EWS-FLI1 cooperates with the IGF system in establishment of the pathology. Therefore, this study provide a crucial explanation of the elevated IGF-1R expression described in 70 to 80% of patients [282] and more strongly support the importance of EWS-FLI1 and IGF system in maintenance of ES malignancy. On the contrary, data from this study indicate CD99 is differentially regulated between ETS-related tumors as CD99 is a target of EWS-FLI1 but not of tERG. As previously demonstrated, within different tumors ETS rearrangements can differentially regulate expression of target genes like the reported KCNN2 [370]. In ES, CD99 represents a validated EWS-FLI1 target as the chimera was found to bind its promoter and EWS-FLI1 forced expression in mesenchymal stem cells induced up-regulation of CD99 [94, 373]. In PCa, CD99 acts as a potential oncosuppressor and it did not show significant differences between tERG-positive and –negative cells both considering binding of ERG to the promoter and transcript levels. Noteworthy, CD99 expression is decreased in PCa in general suggesting that regulation of CD99 may be controlled by other mechanisms, such as promoter methylation as described for CAV1 gene in PCa [374]. In this study, a direct correlation was anyway found between ERG and CD99 proteins both in vitro and in patients. Explanation of this relationship is not easy and it would request
more detailed analysis but putatively suggests that ERG target genes comprehend regulators of CD99, possibly including miRNAs as previously demonstrated in ES [375]. Considering that ERG expression represents a marker of malignancy while CD99 is associated with a benign phenotype in could be speculated that effective relevance of this interaction could be marginal consistently with absence of association between CD99 and survival.

From the clinical standpoint, the ETS/IGF-1R mechanism appears of high interest as it provides basis for a more rationale use of anti-IGF-1R inhibitors in these tumor types. In PCa, the contribution of IGF-1R to prostate carcinogenesis and progression remains controversial, but epidemiological, preclinical and clinical results indicate that IGF-1R over-expression plays an important role in the pathogenesis of CRPC. This evidence, in particular, lead to the enrollment of castration-resistant prostate cancer patients in several clinical trials investigating the effects of IGF-1R inhibitors but the studies evidenced a modest effect of IGF-1R inhibition with a minority of patients experiencing significant benefits. Data from this study demonstrate that only PCa cells expressing the fusion gene and consequently higher levels of IGF-1R display a potential relevant sensitivity to anti-IGF-1R agents. Accordingly, ERG silencing caused a decreased sensitivity to the treatment. These results are in line with previous evidences demonstrating that PARP1 inhibitors blocked ETS-positive but not ETS-negative prostate cancer xenograft growth and reflect more recent evidences showing that treatment with ganitumab, an IGF-1R inhibitor, blocked growth of T2E-positive VCaP but not T2E-negative 22RV1 xenograft models [376]. In addition, ERG silencing determined a decreased sensitivity to abiraterone acetate, consistently with previous observations that T2E-positive PCa patients display a better response to anti-androgen therapy. In clinic, the onset of androgen receptor-linked resistance mechanisms in CRPC patients treated with abiraterone represents an important limitation and the identification of “druggable” target involved in the androgen receptor pathway represent an interesting opportunity to overcome the resistance. In this perspective, different studies have been designed in order to combine abiraterone with targeted agents including Src inhibitors [377] or PI3K pathway inhibitors [378]. The results provide a preliminary in vitro evidence regarding the benefits of a combined treatment of the monoclonal antibody CP-751,871 with abiraterone acetate. Particularly, in VCaP cells association of CP-751,871 with abiraterone acetate gave synergistic effects, supporting the concept of a simultaneous use of two targeted agents to deprive tERG-expressing cells of fundamental pathways that operate in concert to sustain cell proliferation. Overall, data in PCa provide a criterion for patient selection, identifying CRPC patients expressing TMPRSS2-ERG as good responders to anti-IGF-1R treatment. In ES, all of
the patients express the EWS-FLI1 chimera therefore avoiding application of a similar criterion in this tumor type. Anyway, presence of EWS-FLI1/IGF-1R axis provides rationale for combination of anti-IGF-1R agents with the newly licensed chemotherapeutic agent trabectedin consistently with its effects on IGF-1R expression levels. Trabectedin is an alkylator agent particularly toxic in sarcomas bearing translocations. The reason is the recently evidenced capability of DNA binding agents including trabectedin but also DXR, mithramycin, and actinomycin D to alter transcriptional activities of ETS rearrangements attenuating their gene signature. These data introduce a certain level of specificity in the action of conventional agents, depending on the drug itself, the transcription factor and the cellular context. Accordingly, trabectedin and DXR caused detachment of EWS-FLI1 chimera from its target promoters, including *TGFβR2* and *CD99*, while only trabectedin enhanced EWS-FLI1 occupancy on the IGF-1R promoter. Increased EWS-FLI1 binding to specific promoters represent another variable in the mechanism of action of chemotherapeutics and, in addition, appears peculiar of this tumor type. In PCa trabectedin was found to decrease binding of ERG to both *PIM-1* and *IGF-1R* promoters without affecting binding to *CD99* promoter evidencing the cell-specific action of this agent. These data are in line with previous evidences in myxoid liposarcoma where trabectedin but not DXR affected the binding of FUS-CHOP to target promoters [26]. In ES, increased binding of EWS-FLI1 to *IGF-1R* promoter corresponded to higher IGF-1R protein expression providing the rationale for a combined use of trabectedin with anti-IGF-1R agents. Combination of trabectedin with the dual inhibitor IGF-1R/IR OSI-906, a small molecule shown to have anti-tumoral activity against several tumors, gave synergistic effects in the ES cell lines considered in this study. On the opposite side, no synergistic effect was observed in VCaP PCa cells harboring the fusion gene. Use of anti-IGF-1R inhibitors in ES gave satisfactory results at preclinical levels but limited effectiveness in clinic [379]. Use of trabectedin as single agent did not demonstrated significant activity in sarcomas including ES [99]. Considering the paucity of therapeutic choices in ES, these data demonstrate the potential benefit of a combination between trabectedin and anti-IGF-1R agents and provide a novel therapeutic strategy for treatment of ES patients.

Understanding the clinical role of the IGF system represents an important choice to defeat tumor cells because different subtypes of patients may have distinct outcome and may require differential treatment. In this study, expression of different components of the IGF system was analyzed in a primary PCa series taking in account gene fusion presence. Overall, no relevant differential expression was found between tumor and normal cells except for a lower tumor expression of IGFBP-3, consistently with
published data. In addition, as prognostic implications of IGF system components expression are still controversial, association with outcome was evaluated both considering total cases and subgroups of patients harboring or not the fusion gene. Overall, the findings reported in this study support a relationship between high mRNA expression of \textit{IGF-1} and \textit{IGF-1R} and favorable outcome. High expression of \textit{IGF-1R} by immunohistochemistry did not distinguish patients with different prognosis but immunohistochemistry has limited power in terms of antigen quantification. Previous studies regarding \textit{IGF-1R} prognosis value reported similar results in sarcomas and in carcinomas. In ES, lower \textit{IGF-1} circulating levels were found in patients with metastatic disease [380] while in a cohort of 57 patients a relationship was found between high expression of \textit{IGF-1R} and \textit{IGF-1} and favorable prognosis [282]. In breast cancer, higher expression of \textit{IGF-1R} was found in tumor specimens compared to matched control samples [381]. Data in PCa are highly conflicting but, as previously reported, some of them are in accordance with a correlation between elevated \textit{IGF-1R} activity and minor malignancy. Results from this study add another level of complexity because, while \textit{IGF-1} significance was maintained in all the analyzed subgroups, \textit{IGF-1R} was found to influence BPFS in the TMPRSS2-ERG-negative patients while marginal or no association was found in the total cases or TMPRSS2-ERG-positive cases, respectively. Several studies report that TMPRSS2-ERG presence defines patients with different biological and clinical behaviors [191]. For this reason, identification of molecular targets related to androgen-mediated activation of TMPRSS2-ERG has a relevance for the clinical management of PCa. Lesions mutually exclusive with presence or absence of ETS rearrangements have been found laying the basis for the molecular characterization of PCa, often beginning with ETS-positive and ETS-negative subclasses. Mutations and deletions in \textit{PTEN} and \textit{p53} are enriched in ETS-positive tumors while mutations on \textit{SPOP}, \textit{CDH1}, and over-expression of \textit{SPINK1} exclusively occur in ETS-negative tumors defining molecular subtypes of PCa [382]. Recently, molecular alterations in \textit{SPOP} gene were found to define a new subtype of PCa and the prognosis value of SPOP was statistically more significant in the subgroup of patients without the fusion gene [179]. Accordingly, the obtained results identify the subgroup of ETS-negative and \textit{IGF-1R} low-expressor patients as a group with a particularly poor biochemical progression free survival. \textit{IGF-1R} could thus represent a useful biomarker for patients not harboring the fusion gene alongside the parameters already used in clinic. IGF system sustains tumor cell proliferation, protects cells from apoptosis and DNA damage but also favors differentiation [218], depending on the cellular context. Overall, the presented results indicate \textit{IGF-1R} drives different effects depending on the presence or absence of TMPRSS2-ERG. In case of PCa, it has been
already reported that TMPRSS2-ERG regulates several pathways including differentiation [383]. Indeed, it has been recently demonstrated that TMPRSS2-ERG blocks luminal cell differentiation driving proliferation [384]. As a consequence, it could be speculated that when TMPRSS2-ERG is expressed, IGF-1R acts in a less differentiated phenotype where drives proliferation while IGF-1R activity turns toward differentiation in presence of TMPRSS2-ERG and a more differentiated context. These conclusions could be in accordance with a recent in vitro study showing that IGF-1R stimulation induces differentiation in non-malignant cells while induces proliferation in malignant cell models [385] further supporting the importance of the cellular context for IGF-1R activity.

Identification of biomarkers in PCa represents an urgent need. With this perspective and to get more insight regarding its value in PCa, CD99 prognostic relevance was evaluated. Prognosis value of CD99 has been recently demonstrated in multiple myeloma, where its expression correlates with longer overall survival [386], and in osteosarcoma, where low expression of this molecule correlates with poor outcome [387], while no previous information was available in PCa. In this study, despite a little trend suggesting a correlation between CD99 expression and a better biochemical free progression free survival, no clinical relevance for CD99 was found in the field of prognostic biomarkers. In accordance to preliminary data indicating a role of CD99 in metastasis, the data suggest that CD99 loss could represent a relevant event in influencing invasiveness processes as supported by the association between CD99 and positivity of margins.

In conclusion, this study demonstrates that IGF-1R is an important target of tERG and EWS-FLI1, and that this interaction has relevant translational implications both in PCa and in ES. In PCa, it leads to a higher IGF-1R expression both in cell lines and in patients providing the rationale for treating the subpopulation of patients expressing T2E with anti-IGF-1R agents especially in combination with abiraterone acetate. In ES, trabectedin enhanced binding of EWS-FLI1 to the IGF-1R promoter, which resulted in increased IGF-1R expression, suggesting the criterion for development of a therapy that combines trabectedin with anti-IGF signaling agents. CD99 is differentially regulated between PCa and ES, beside ETS consensus sequences are present in its promoter suggesting further analysis are required. In addition it did not display prognostic value in PCa while IGF-1R expression discriminates patients with different outcome inside the subgroup of cases negative for the fusion gene.
Bibliography


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