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Study of the interactions between CD99, immunologic microenvironment and microRNAs and their prognostic significance in human osteosarcoma

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“A Model must be wrong, in some respects,

else it would be the thing itself.

The trick is to see where it is right.”

(Henry A. Bent)

Abstract

Osteosarcoma is the most frequent primary tumor of the bone, characterized by an aggressive and metastatic potential. Few improvements were achieved in term of therapies in the last two decades. Understanding mechanisms underlying chemosensitivity of tumor cells and the discovery of new prognostic factors could improve patients' outcome.

In this study, expression of CD99 had been evaluated by IHC in 100 OS biopsies, discovering the potential of CD99 as a predictive biomarker of ifosfamide response. The importance of CD99 in ifosfamide sensitivity was also studied in osteosarcoma cell lines. For the same OS specimens, expression of immune infiltrate markers and their correlation with CD99 has been studied. For understanding the regulation of CD99 in osteosarcoma, miRNA mediated post-transcriptional regulation has been examined. Finally, a small RNA sequencing pilot study has been conducted on 14 OS samples, with the aim to identify a miRNome able to distinguish at diagnosis patients with high probability to relapse. This signature has been validated by qPCR in a larger OS cohort.

The predictive and prognostic role of CD99 in OS has been reported. In clinical samples, CD99 acts as a predictive biomarker for ifosfamide response. CD99 is associated with an increased sensitivity in OS cell lines, corresponding to a greater apoptosis rate. CD99 tumoral expression correlates to an enrichment in CD8+ and CD68+ cells in OS samples. Taken together these data confirm a role of CD99 in ifosfamide sensitivity in osteosarcoma, thus CD99 is able to increase both the cytotoxic and the immunomodulatory effects of ifosfamide, making osteosarcoma cells more sensitive. The study regarding post-transcriptional regulation of CD99 identifies hsa-miR-330-3p as a modulator of CD99 expression. Moreover miR-330-3p affects downstream effector of CD99, like ROCK2, leading to a modulation of migration capabilities. Finally, a 3 miRNAs signature (hsa-miR-99b-3p, hsa-miR-130b-5p and hsa-miR-139-5p), identified by small RNA sequencing, discriminates patients who remained disease-free from those who relapsed. Enrichment pathways analyses show the involvement of these miRNAs in immunological processes and cellular metabolism, underlining the importance of these mechanisms in the pathogenesis and response to therapy in OS.

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1. INTRODUCTION

1.1 OSTEOSARCOMA

1.1.1 General aspects

Osteosarcoma is the most common primary malignancy of bone, despite it is a rare tumor, with a worldwide incidence of 4-5 cases per million people per year. In fact, osteosarcoma cases represent only 0.2% of all cancers diagnosed (Beckingsale, 2010).

It has a high social impact because it affects predominantly adolescents and young adults and it is characterized by aggressive features like high metastatic potential and incidence of local recurrence. There is a minor second peak of incidence in patients older than 60 years. (Savage, 2011).

The first peak corresponds to an increased bone growth typical of puberty with a mean age of patients of 16 years. The onset in elderly patients is secondary to a previous radiation expositions or bone abnormalities, like Paget's disease (Savage, 2011; Mirabello, 2009)

Osteosarcoma affects both males and females, with a ratio of 1.6:1; this tumor develops a little bit earlier in female rather than in male, reflecting the difference in the beginning of puberty existing between the gender.

In young patients, osteosarcoma occurs mainly in the metaphysis of long bones, such as the distal femur, proximal tibia, and proximal humerus: these are bone districts characterized by rapid bone growth, corroborating the relationship between osteosarcoma onset and bone development during puberty. Anatomic sites change with age, in elderly patients osteosarcoma affects flat and axial bones, confirming a different pathogenesis in the two classes of patients (Hayden, 2006).

Osteosarcoma presents a wide range of histological subtypes; the WHO recognize the following variants: classic or conventional, small cells, telangiectatic, periosteal and parosteal. All these variants have in common an uncontrolled proliferation of mesenchymal-derived cells able to produce osteoid matrix (Marina, 2004).

Classic osteosarcoma represents the most common histological types and it is described as formed by cells with different morphology, with pleomorphic and hyperchromatic nuclei. Classic

osteosarcoma can be divided in osteoblastic, chondroblastic and fibroblastic, based on the predominant type of matrix secreted by tumoral cells (Klein, 2006; Picci, 2014).

Osteosarcoma is also classified by anatomic location and tumor staging. Most of the cases occurs in the intramedullary part of the bone, while only 5% develops on the bone surface.

Staging system used by Musculoskeletal Tumor Society is the Enneking system and it is based on:

- tumor grade: high grade (I) or low grade (II)
- tumor extension: intra-compartmental (A) or extra-compartmental (B)
- presence of distant metastasis (III)

The staging of a tumor is a measure of the tumor growth and diffusion and considers of its microscopic aspect (Picci, 2007).

1.1.2 Etiology

The etiology of osteosarcoma is not well understood yet.

It is possible that factors connected to the growth play an important role in osteosarcoma etiology since this tumor mainly occurs during puberty, a period characterized by a rapid development and bone remodeling. Many studies confirm an association between taller stature and increased risk to develop osteosarcoma, because of a more rapid growth rate during the puberty of taller people in respect to the others. Also, high birth weight could be a risk factor for this type of tumor. These association could be explained studying some factors involved in high birth weight, such as growth factors and hormones (Reviewed in Mirabello, 2011).

The IGF system plays a key role in the growth hormone signaling pathways, in addition to its function as normal bone growth and development regulator. Also, it was demonstrated that a SNP in IGF-IIR gene is associated with an increased risk of osteosarcoma onset (Scotlandi, 2008).

There are few knowledges about environmental exposition and osteosarcoma because it is difficult to conduct association studies due to the rarity of the tumor and the limited samples size. The only validated evidence concerns the exposition to radiation and to beryllium oxide (Hayden, 2006).

Several familiar syndromes predispose to osteosarcoma, such as hereditary retinoblastoma, Li-Fraumeni syndrome, Rothmund-Thomson syndrome, Werner syndrome and Bloom syndrome.

These diseases are extremely rare and due to highly penetrant germline mutations. These association studies could provide some important bases for understanding osteosarcoma etiology (Kansara, 2007).

Some authors suggest a viral etiology, based on the evidence that some osteosarcomas show SV-40 virus sequences. Nevertheless, there are no clinical data supporting that viruses are important for the onset of this tumor (Longhi, 2006).

1.1.3 Genetic and molecular bases of osteosarcoma

Osteosarcoma is typically aneuploid and lacks specific chromosome translocations, characterizing most of sarcomas, like Ewing Sarcoma (Kansara, 2007).

Osteosarcoma is known for its high genomic instability and complexity of mutations and chromosomic rearrangements, proving objects for several genetic and genomics studies with the common aim to understand the cause of this catastrophic scenario.

Several techniques were used for studying mutations and rearrangements at level of both single gene and entire chromosome, such as karyotyping, comparative genomic hybridization (CGH), fluorescence in situ hybridization, quantitative PCR, single-strand conformation polymorphism analysis, genome-wide association studies using single-nucleotide polymorphisms (SNPs). Thanks to these studies, it was possible to identify some chromosomic regions that are mainly affected by mutations. Numeric abnormalities include gain of chromosome 1 and loss of chromosomes 6, 9, 10, 13 and 17; instead, structural rearrangements involve chromosome 11, 19 and 20 (Kansara, 2007; Hayden, 2006). These common rearrangements could help to better comprehend and study genes involved in osteosarcoma development and progression. Thanks to these worth cytogenetic studies and genomic analyses, some important genes were identified, like RB, mapping on chromosome 13p, and TP53, mapping on 17p (Kansara, 2007; Hayden, 2006). The importance of these two genes are also supported by the onset of osteosarcoma in patients affected by hereditary retinoblastoma and Li-Fraumeni syndrome.

Oncosuppressor retinoblastoma, Rb, is one of the best characterized gene involved in osteosarcoma. It is located on chromosome 13q14 and it encodes for a 110 kDa protein belonging to the family of pocket proteins. pRb negatively regulates cell cycle progression from G0/G1 to S phase and it is deregulated in all cancers. During G1 phase, pRB binds and suppresses E2F1, E2F2 and E2F3 transcription factors. Phosphorylation of Rb by cyclin-dependent kinases CDK4, CDK6 and CDK2 leads to inactivation of Rb, release of E2F transcription factors and subsequently transcription of gene necessary to cell cycle progression,

such as cyclin A, D and E. Cyclin-dependent kinase inhibitors (CDKI), p16INK4a and p15INK4b, prevent cyclin D and CDK4 functions. In this way they inhibit cell cycle progression blocking G1/S transition. 70% of sporadic osteosarcoma cases present Rb mutations, homozygous deletions are observed in 23% of cases, instead punctiform mutations in just 6% (Kansara, 2007).

TP53 gene encodes for a transcription factor involved in cell cycle regulation, DNA damage response and apoptosis. After a DNA damage, p53 upregulates p21 transcription. p21 binds complexes formed by cyclin E/CDK2 or Cyclin D/CDK4/6, resulting in a transient arrest of cell cycle. If the damage cannot be repaired, prolonged activation of p53 leads to apoptosis. Germline mutations are observed in patients affected by Li-Fraumeni syndrome. These patients are predisposed to develop different tumors, included brain, breast, soft tissue, adrenocortical tumors, leukemia and osteosarcoma. Osteosarcoma is the second more frequent tumor developed in Li-Fraumeni patients, with an incidence of 12%. In sporadic osteosarcoma, TP53 is mutated in 60% of cases. Different mechanisms may lead to inactivation of p53, such as allelic loss (70-80%), punctiform mutation (20-30%) and genic rearrangements (10-20%) (Kansara, 2007).

Murine models of osteosarcoma corroborate the importance of p53 in osteosarcoma development. There are different models with a predisposition to osteosarcoma, bringing p53 mutation or deletion. Instead, Rb mutations alone do not predispose to osteosarcoma development in mouse models (Janeway, 2010).

Furthermore, several aberrations are observed in p53 regulators and effectors, like MDM2 and CDKI.

MDM2, localized on 12q13-q14 chromosome, encodes for a protein able to bind pRB and inhibit p53. There is a feedback loop between p53 and MDM2, because p53 controls MDM2 transcription and MDM2 can block both p53 transcription activity and its transport from the cytoplasm to the nucleus. MDM2 also functions as E3 ubiquitin ligase that recognizes the N-terminal trans-activation domain (TAD) of p53, causing its degradation. MDM2 is amplified in 17% of osteosarcoma and its expression is associated with the development of local recurrence and distal metastasis (Kansara, 2007).

CDKIs inhibit cell cycle progression and the inactivation of these inhibitors could lead to cell transformation and immortalization. INK4A plays a crucial role in the regulation of G1 phase: the gene encodes for two different transcripts, p16INK4a and p14ARF. p16INK4a interacts with and blocks MDM2: inhibition of MDM2 leads to a stabilization of p53 and subsequently to the

arrest of cell cycle and apoptosis. Homozygous deletion of INK4a is noted in 10% of osteosarcoma, correlating with a poor prognosis (Kansara, 2007).

The complexity of osteosarcoma karyotype could reflect its pathogenesis. This complexity could be due to the cycle of chromosomic fusion and break, deriving by telomeric shortening. Telomers are nucleoprotein structures that protect chromosomic extremities. Telomeric length is one of the check points controlling cell proliferation: when telomers are too short there is an activation of a p53-dependent senescence pathway. Tumoral cells bypass this checkpoint through keeping the correct telomeric length. About 85% of tumors activate a specific enzyme, called telomerase. In the rest of cancers, telomer elongation occurs through a recombination-based mechanism named ALT (Alternative Lenghtening of Telomers). About 50% of osteosarcoma depends on ALT for the maintenance of telomer functions (Marina, 2004).

1.1.4 Osteosarcoma and osteoblastic differentiation

In the last few years osteosarcoma has been considered as a differentiation disease. In fact, epigenetic and genetic alterations interrupt the normal bone differentiation. Bone marrow stem cells are capable to differentiate into osteoblasts, chondroblasts, adipocytes, muscular cells and neural cells. Bone differentiation is an intricate and fine-tuned process influenced by environmental and endogenous factors. Several pathways are involved in this process, like WNT, BMPs, FGF, IGF. Different stages of osteoblast differentiation are characterized by expression of specific marker and progressive loss of proliferating ability (Basu-Roy, 2013).

Several evidences support the involvement of bone differentiation in osteosarcoma pathogenesis.

Osteosarcoma is formed by a heterogeneous population of cells, some of which show stem-cell like features. Cancer stem cells are characterized by asymmetric division, self-renewal and chemoresistance (Wagner, 2011). Gibbs and colleagues isolated a subpopulation of cell able to form spherical colonies, named sarcosphere; these cells express typical stemness markers, like Oct3/4, Nanog e STAT3 (Gibbs, 2005). The presence of osteoid matrix suggests that osteoblast progenitors may incur in neoplastic transformation (Dani, 2012). In fact, osteosarcoma cells only express early marker of bone differentiation like ALP and collagen (Wagner, 2011).

Loss of differentiation has a prognostic relevance: well-differentiated tumors are classified as low grade and have a significantly better prognosis than the undifferentiated ones. Osteoblastic differentiation is antagonist to oncogenic process: the last stage of differentiation is the cell-cycle exit regulated by the transcription factor Runx2 (Thomas, 2004).

Runx2 belongs to a family of transcription factor characterized by some conserved motifs: a DNA binding domain, a protein-protein interaction domain, one with ATPase activity. Runx2 is involved in several steps of bone differentiation. Runx2 could be co-activated by Rb; also, Runx2 coordinates cell cycle exit through activation of p27KIP1. Both Rb and p27KIP1 are lost in osteosarcoma, so Runx2 activity is frequently interrupted in this tumor leading to the loss of differentiated phenotype (Thomas, 2004).

1.1.5 Clinical aspect: from diagnosis to therapy

Patients affected by osteosarcoma complain pain and swelling. Pain may be prolonged for weeks or months, also during the sleep. Swelling appears successively and the interested area becomes hard to tact and shows signs of hypervascularity. Often the patients can lose the functionality of the limb and frequently it is possible to attend to pathological fractures (Klein, 2006).

Despite most of the patients show the symptomatology of the primary tumor, some others could complain different symptoms like dyspnea, chest pain, hemoptysis and cachexia correlated to the metastatic disease.

The first diagnostic step consists in a X-ray radiography of interested bone showing the osteoid matrix and the presence of tumoral mass. Diagnosis is then confirmed by biopsy (Beckingsale, 2010).

Even if surgery is a milestone of osteosarcoma treatment, alone it gives only local control of the disease.

The percentage of survival has been improved in the last years, thanks to introduction of chemotherapy. The survival rate has increased up to 70% in patients with no metastatic disease. Unfortunately, a large fraction of patients show metastases at diagnosis and the survival rate of these patients is about 25% (Hayden, 2006).

Adjuvant therapy consists in surgical removal of tumor and post-operatory chemotherapy. The introduction of this kind of treatment for osteosarcoma is one of the biggest success of modern cancer treatment (Kansara, 2007). Neoadjuvant therapy was introduced by Rosen in 1978. Preoperatory chemotherapy is necessary to reduce tumoral mass and control metastasis. This is the last improvement in osteosarcoma cure.

Doxorubicin and methotrexate were the first two antineoplastic drugs introduced for osteosarcoma treatment. Other drugs like vincristine, bleomycin, actinomycin-D were immediately abandoned due to insufficient effectiveness. Cisplatin and ifosfamide were added in

chemoprotocol many years later (Longhi, 2006). Methotrexate, cisplatin and doxorubicin have the highest antitumoral activity in osteosarcoma when used as single agents. Combination of these drugs shows synergic effect, leading to a more efficient therapeutic regimen (Beckingsale, 2010).

Methotrexate (MTX) is an antagonist of folic acid, which differs from this one for a substitution of an amminic group to a hydroxilic group in the pteridine ring. MTX binds catalytic site of dihydrofolate reductase (DHFR), interfering with the synthesis of folic acid. Absence of this co-factor interrupts synthesis of tymidilate, purinic nucleotides, serine and threonine amino acids. Thus, MTX inhibits DNA, RNA and protein formation.

Low dose MTX enters cells by an active transport system via the Reduced Folate Carrier (RFC); high dose MTX passively diffuses into the cells. In osteosarcoma, resistance to MTX is mediated by DHFR overexpression and RFC downregulation (Guo, 1999).

Antracyclinic antiobiotics are the most useful antineoplastic drugs ever developed. Doxorubicin (DXR) belongs to this class of drugs. The mechanism of action of doxorubicin is complex and the drug exerts its toxicity through different pathways. Doxorubicin has high affinity for DNA and acts as aspecific intercalating agent, binding and stabilizing ternary complex formed by DXR, topoisomerase II and DNA. DXR also binds cellular membrane altering the fluidity and ion transport (Chou, 2006). In osteosarcoma, the main resistance mechanism is ABCB1 overexpression, leading to an active efflux of DXR outside the cell (Serra, 1995). Overexpression of ABCB1 is also associated with poor prognosis in this tumor (Serra 2003; Serra, 2006).

Cisplatin was introduced in osteosarcoma treatment in the late 1970s. Cisplatin (CDP) is an inorganic metallic compound, able to interact and damage DNA, inducing cell death through activation of apoptotic pathway. Cisplatin needs to be activated by aquation: two chloride atoms are displaced by two molecules of water. The cytotoxic effects are due to DNA interaction and formation of abduct between platin and N7 of purinic bases. Cisplatin induces different kind of bonds: between adjacent bases, inter- and intra-strands, DNA-protein. These abducts change helix conformation which is recognized by DNA damage repair complexes, like mismatch repair complex (MMR). The final effects of cisplatin are cell cycle arrest, inhibition of transcription and activation of apoptotic cascade. Similarly to what happens for DXR and MTX, cells display different mechanisms of resistance to cisplatin. In osteosarcoma, an increased activity of glutathione and glutathione S-transferase (GSH/GST) detoxification system is responsible for CDP resistance (Siddik, 2003).

Thanks to the introduction of MAP (combination of MTX, DXR and CDDP) therapy, overall survival of patients with localized disease were risen to 65%.

Other two drugs can be used in combination with MAP therapy: ifosfamide and etoposide.

Ifosfamide was introduced in osteosarcoma therapy in the ‘80s. Ifosfamide is an alkylating agent belonging to the class of oxazaphosphorine. It is administered as a prodrug and it undergoes to liver activation by cytochrome P450. The metabolism produces several active compounds, like nitrogen mustard, 4-hydroxyifosfamide and acrolein. (Giraud, 2010). The cytotoxic effect depends on the DNA crosslink through formation of covalent bonds. Little is known about the specific mechanism of action of oxazaphosphorines. Ifosfamide and its active compounds are hydrophilic and their transport inside the cell is mediated by active transporters like breast cancer resistance protein (BCRP), some members of multidrug resistance protein (MRP1, MRP2, MRP4), organic anion transporter (OAT) and organic cation transporter (OCT). Resistance to ifosfamide is due to active efflux thanks to upregulation of the transporters; an increased detoxification of oxazaphosphorines metabolites through aldehyde dehydrogenase (ALDH), glucose-6-phospate dehydrogenase (G6PD), GSH and GST system; anti-apoptotic response to cellular damages and increased repair of DNA (Zhang, 2005). Nothing is known about the exact mechanism of resistance in osteosarcoma.

Etoposide is often coupled with ifosfamide in sarcoma treatment; etoposide alone shows a low cytotoxic effect in OS cells. This drug is a podophyllotoxin that interacts with topoisomerase II, forming a complex DNA-enzyme (Gill, 2013).

Unfortunately, no progress has been made in the therapy regimens over the last 30 years. Probably a better comprehension of molecular pathogenesis of OS could identify important deregulated pathways, giving the possibility to develop new targeted therapies.

Only one new drug is approved for treatment of localized disease: MTP-PE. MTP-PE is a synthetic lipophilic analogue of muramil dipeptide; it is able to selectively activate macrophages and monocytes and induces secretion of some cytokines, such as IL-6 and TNF-α. Clinical studies show that introduction of MTP-PE decreases local relapse rates and improves survival rate up to 78% (Meyers, 2008).

1.1.6 Prognostic factor

In the last years, a large number of studies have been conducted with a common aim: identify novel biomarkers. Prognostic and predictive biomarkers are useful tools for improving cancer

treatment. Unfortunately, there are discordant opinions about the value of some factor identified, probably due to lack of uniformity in techniques used and cohort selection.

Age at diagnosis, anatomic location and histological subtypes, volume of tumor, low chemoresponsiveness, type of surgery and chemoprotocol are clinical factors often associated with a worse prognosis (Longhi, 2006).

Thanks to neoadjuvant chemotherapy, it is possible to measure the tumor responsiveness to chemotherapy, evaluating necrosis grade of the tumor after surgical removal. Tumoral necrosis is a widely-accepted prognostic factor. A high percentage of necrotic cells significantly correlates with a better prognosis, both in term of event-free survival and overall survival (Gill, 2013).

Several biological factors are considered in multiple studies and show antithetical value.

High level of alkaline phosphatase and lactate dehydrogenase seem to correlate with worse prognosis (Bacci, 2006).

P-glycoprotein (ABCB1) is the only biological factor recognized for its prognostic relevance. Several studies show that the overexpression of this ATP-dependent pump is a negative prognostic factor in osteosarcoma patients. Based on expression level of ABCB1, patients are stratified before the beginning of pre-operatory chemotherapy and follow different schedule of drugs administration (Scotlandi, 1996; Serra, 2003).

1.2 The molecule CD99

1.2.1 General aspects

CD99 was described as an antigen recognized by 12E7 monoclonal antibody for the first time in 1979 by Levy and colleagues. They demonstrated that CD99 is highly expressed in cortical thymocytes and acute lymphoblastic leukemia (ALL), but absent in medullary thymocytes and in chronic myeloid leukemia (CML) and chronic lymphatic leukemia (CLL) (Levy, 1979). This protein is codified by MIC2 gene, the first pseudoautosomal gene ever identified (Goodfellow, 1980, Goodfellow, 1983; Goodfellow, 1986; Smith, 1993). MIC2 is 50 kbp long and it is composed by ten exons; it is oriented toward the centromers and maps in the pseudoautosomal region of sexual chromosomes (Xp22.3- Xpter and Yp11-Ypter). MIC2X and MIC2Y genes are strictly correlated to each other and their products are biochemically equal. Moreover, MIC2X is not subjected to chromosome X inactivation, maintaining the same genic dosage in males and females (Goodfellow, 1984). Product of MIC2 gene does not show any homology with known proteins except for Xg, an erythrocyte antigen. The homology covers the 48% of the amino acid sequences, probably due to a duplication of an ancestral gene resulting in the formation of these two genes (Fouchet, 2000). CD99 is a glycoprotein, localized into cell membranes, with a molecular weight of 32 kDa. CD99 has one extracellular domain of 100 amino acids highly O-glycosilated, a hydrophobic transmembranous domain of 23 residues and a C-terminal intracellular portion of 38 amino acids. The intracytoplasmatic domain has a hairpin structure bond to the membrane by two flexible handles; a consensus site for PKC is present in this domain, that includes a serine residue in position 168 (Alberti, 2002; Kim, 2004).

1.2.2 CD99 and physiological role

CD99 has a ubiquitous expression in normal cells and tissues. Immunohistochemistry analyses of several human tissues report a high expression in: cortical thymocytes, a subgroup of lymphocytes presents both in bone marrow and lymph nodes; endothelial cells of blood vessels; Langerhans cells in pancreas; testis cells such as Sertoli and Leidig cells; ovary granulosa cells and ependymal cells of brain and spinal canal (Ambros, 1991).

CD99 is expressed in all leukocyte cell types in thymus, bone marrow and peripheral blood and its expression is inversely correlated to differentiative stage in lymphatic and granulocytes lineage (Dworzac, 1994). Moreover, CD99 is expressed at high level in B- and T- memory cells

(Park, 1999). Recently, a colocalization CD99-MHC I/II has been demonstrated in immunological synapsis, suggesting an involvement of CD99 in immune response (Pata, 2011).

Natural ligand of CD99 is still unknown, the use of monoclonal antibodies (mAbs) is necessary for studying downstream effectors. The binding CD99-mAb activates the signal cascade. CD99 is involved in adhesion and apoptosis of T cells: activation through mAbs interferes with the rosette formation and induces externalization of phosphatidyl-serine in thymocytes but not in mature T cells (Aussel, 1993). Moreover, triggering of CD99 induces homotypic aggregation and apoptosis in Jurkat cells and in CD4+ CD8+ cortical thymocytes, but not in medullary thymocytes or other T cells (Bernard, 1995; Bernard, 1997). The apoptotic process is Fas/CD95 independent and it is not characterized by DNA fragmentation (Bernard, 1997). Homotypic aggregation induced by CD99 in T cells does not activate LFA-1/ICAM-1 signaling pathway and it is strictly associated to actin remodeling and it depends on Ca2+ release and ERK activation (Kasinrerk, 2000; Hanh, 2000). Furthermore, activation of CD99 induces aggregation in B cell through a LFA-1/ICAM-1 dependent mechanism (Hanh, 1997). CD99 acts as a co-stimulator of T-cells activation in peripheral blood, favoring proliferation and expression of typical markers of activated T cell (Waclavicek, 1998; Wingett, 1999). CD99 influences the maturation process in thymocytes facilitating the interaction between TCR and MHC complexes through modulation of intracellular trafficking. Stimulation of CD99 induces the transport of TCR and MHC to plasmatic membrane, resulting in a higher expression of these proteins at cellular junctions. This process leads to a positive selection of thymocytes, because cells able to recognize complex MHC/Antigen are driven to proliferation, maturation and migration (Choi, 1998). Yoon and colleagues confirmed the role of CD99 in cellular trafficking, demonstrating that CD99 activation in Jurkat cells induces transport of some proteins (TCR, LFA-1, ICAM-1, CD5 and GM1) to plasma membrane through the remodeling of actin cytoskeleton (Yoon, 2003). So, CD99 may have a physiological role in maturation and activation of T cells.

CD99 is also involved in the process of transendothelial migration. The interaction between CD99 molecules on the surface of endothelial cells and of monocytes is fundamental for the diapedesis (Shenkel, 2002; Lou, 2007). Furthermore, CD99 is an important factor of inverse transmigration, a process similar to diapedesis, through which activated dendritic cells migrates from inflammatory sites to lymph nodes (Torzicky, 2012).

1.2.3 CD99 and cancer

CD99 is expressed in a wide range of cancers and its role depends on the cellular context. In fact, CD99 may act as oncogene or a tumor suppressor gene.

In hematological malignancies an oncogenic role of CD99 is described in T-cell of acute lymphoblastic leukemia (T-ALL), where its expression correlates with minimal residual disease (Levy, 1979; Dworzac, 2004). Antigen CD99 is expressed in cells of lymphoblastic lymphoma and anaplastic large cells lymphoma (Sung, 2005; Buxton, 2009). In diffuse large B cell lymphoma CD99 is associated to an advanced stage of the disease and correlates with unfavorable prognostic factors (Lee, 2011). Dworzac and colleagues demonstrated that expression pattern of CD99 is maintained despite the neoplastic transformation in B cell lymphoblastic leukemia (Dworzac, 1999). Also, CD99 is expressed in solid tumors, such as embryonal rhabdomyosarcoma (Kovar, 1990; Ambros, 1991; Ramani, 1993), synovial sarcoma (Fisher, 1998), mesenchymal chondrosarcoma (Granter, 1996) and ovaric carcinoma (Cho, 2006). CD99 positively correlates with more malignant characteristics in melanoma (Wilkerson, 2006). In breast cancer, CD99 is highly expressed but it has no prognostic relevance (Wilkerson, 2006; King, 2007). Gastrointestinal and pulmonary neuroendocrinal tumors show a heterogeneous expression of CD99 and the percentage of positive cells significantly correlates with tumor invasion and metastasis formation (Pelosi, 2000). CD99 is expressed at high level in tumors of central nervous system and brain, like ependymoma, astrocytomas and glyomas (Mahfouz, 2008; Ishizawa, 2008; Seol, 2012). CD99 seems to be involved in cellular migration in astrocytomas and its expression is increased in more malignant tumors (Urias, 2014). Among several cancers expressing CD99, Ewing sarcoma (ES) and neuroectodermic primary tumors show the highest level of CD99, which is considered as a diagnostic marker for these diseases (Kovar, 1990; Ambros, 1991). CD99 has an oncogenic role in ES and its expression promotes proliferation and migration (Kreppel, 2006). Silencing of CD99 reduces tumorigenic and metastatic abilities *in vitro* and *in vivo*. Also, loss of CD99 leads to neural differentiation through expression of differentiative markers, such as H-NF and β3-tubulin, and neurites formation (Rocchi, 2010). In Ewing Family Tumors (EFT), the treatment with blocking mAbs direct to CD99 induces homotypic aggregation and great rate of apoptosis, reduces anchorage-independent growth and tumorigenesis *in vivo*. Downstream effects include cytoskeleton remodeling and do not involve protein kinases pathways (Sohn, 1998; Scotlandi, 2000; Cerisano, 2004). Combined treatment of mAbs and doxorubicin or vincristine shows addictive effect (Guerzoni, 2015).

On the other hand, several tumors show a low expression of CD99, suggesting an oncosuppressive role of this antigen. In gastric adenocarcinoma, loss of CD99 is associated to de-differentiation of neoplastic cells (Jung, 2002). Also, in pancreatic neuroendocrinal tumor, high expression of CD99 is associated to a better outcome (Maitra, 2003; Goto, 2004). Recent

studies have been demonstrated the oncosuppressive role of CD99 in multiple myeloma: CD99 level positively correlates with prognosis (Shin, 2014). In Hodgkin lymphoma, CD99 is not expressed unlike in normal B immature cells. Loss of CD99 is crucial for the formation of large tumoral cells typical of this neoplasia (Kim, 1998; Kim, 2000). CD99 acts in osteosarcoma as an oncosuppressor gene. CD99 is expressed in normal osteoblast and not in OS cells (Barteaux, 2005). CD99 expression favorites ECM adhesion, inhibits migration and anchorage-independent growth *in vitro*, reduces tumorigenic and metastatic potential *in vivo*. Ectopic expression of CD99 induces the formation of a complex with caveolin-1 and c-Src, leading to a decreased activity of the kinase (Manara, 2006; Scotlandi, 2007). Also, forced expression of CD99 in OS cells stimulates the recruitment of N-cadherin and β-catenin to cellular junctions and induces cytoskeleton remodeling. Moreover, CD99 inhibits ROCK2 signaling, crucial for OS cell migration (Zucchini, 2014).

1.3 microRNA

1.3.1 General aspects

miRNAs belong to the class of non-coding RNA. They are 19-25 nucleotides long, so they are defined as small non-coding RNAs. miRNAs regulate gene expression through the interaction between their seed sequence and mRNA target. Usually the pairing is not perfect and occurs in the 3'UTR of mRNA target. Binding of miRNA to its target induces translation repression and mRNA degradation. There is some exception to canonical mechanism of action of miRNAs: the binding may involve the 5'UTR or other region of mRNA target; pairing could be perfect; it's possible to assist to target up-regulation. miRNAs belong to the most abundant class of transcriptional regulatory molecules, involved both in physiological and pathological processes (Di Leva, 2014). The first miRNA was described in 1993 when two independent studies in *C. elegans* demonstrated that heterochronic gene lin4 does not codify for a protein but for 2 small RNA, one 22 nucleotides long and its precursor of 61 nucleotides. These studies also showed a complementarity between lin4 and the 3'UTR of lin14. These small RNAs were considered a proper method in nematodes for regulating larval development and were named small temporal RNA (stRNA). In 2001, RNAs with similar features were described in other organisms. Then, a big class of small-RNA with regulatory activity were universally recognized as microRNAs (Di Leva, 2006).

1.3.2 miRNAs biogenesis and mechanism of action

miRNAs biogenesis starts from the transcription of pri-miRNA operated by RNA pol II. Like all Pol II transcripts, pri-miRNAs have a 7-methyl-guanosine cap at 5' extremity and a poli(A) tail at 3'. Genes encoding for miRNA have different location into the genome: in intergenic regions, in the antisense strand in respect to the near gene, in intronic regions. For the first two cases, it is frequent to find cluster of pri-miRNA and the transcript usually is polycistronic. When the miRNA is located into an intron, its expression is associated to the expression of the gene. pri-miRNAs have the same hairpin secondary structure, independently by genome location, with exception for miRNA located in intronic sequences.

The hairpin is processed by a proteic complex, called the microprocessor, formed by the endonuclease Drosha and its cofactor DGCR8. Drosha makes an asymmetric cut on both strands of the hairpin structure near the terminal loop. The product is a pre-miRNA presenting a 5'

phosphorilated and a 3' hydroxylated with an overhang of 2 nucleotides. DGCR8 discriminates the substrate thanks to its two dsRNA binding domains (Gregory, 2005).

An alternative biogenesis mechanism is Drosha-independent and concerns to miRtrons, the miRNA located into intron of a coding mRNA; their processing is operated by the splicing machinery.

pre-miRNAs are transported into the cytoplasm by Exportin5 (Exp5/RanGTP). Exp5 forms a complex with RanGTP and the pre-miRNA. This interaction depends on miRNA structure and it is not influenced by pre-miRNA sequence. The complex Exp5/RanGTP/pre-miRNA reaches the cytoplasm through nuclear pores. GTP hydrolysis mediates pre-miRNA release. The pre-miRNA is processed into a 18-33 nucleotides duplex by another RNase III, Dicer-1, and its cofactor, TRBP. Dicer-1 is highly conserved among the species and is formed by several domains: one for dsRNA binding; one responsible of 3' extremity binding of small RNA, called PAZ; other domains with ATPase and helicase activities. PAZ domain interacts with 3' overhang and the dsRNA binding domain with the duplex. pre-miRNAs are correctly placed into Dicer thanks to the presence of the loop and its dimension. Dicer cuts the pre-miRNA, forming a RNA duplex of 21 bp. ds-miRNA are characterized by a phosphorylation at 5' extremity and a 2 nucleotides overhang at the 3'. (Di Leva, 2006; Meister and Tuschl, 2004).

ds-miRNAs are incorporated into miRISC complex, which is responsible of miRNAs activities. The catalytic core of the complex is formed by Ago proteins. Argonaute family proteins are quite similar to RNase H, with a PAZ domain and a PIWI domain, and nuclease activity responsible of target cleavage. After the bond with the complex, the two strands are unwound thanks to the helicase activity of miRISC. One strand remains associated to the complex, this is the mature miRNA. The other strand, called passenger strand, is degraded. The choice of which strand is incorporated depends on thermodynamic instability and weaker base pairing at the 5' end relative to the other strand (Di Leva, 2006). Mature miRNA is now able to regulate gene expression at post transcriptional level. Interaction miRNA-mRNA occurs at the 5' end of miRNA where the seed sequence is located and a complementary site usually sited in the 3' UTR of the mRNA target. Seed sequence is essential both for target selection and regulation. When the complementarity between miRNA seed sequence and mRNA target is perfect, miRISC cleaves the target. Imperfect pairing leads to a translation block at ribosomal level. It may happen that miRISC complexed to the target is carried into P-bodies, which are cytoplasmatic foci with storage function (Lynam-Lennon, 2009). Other regulation mechanisms consist in destabilization of mRNA target by de-capping or de-adenylating (Filipowicz, 2008). miRNAs regulate several physiological functions, such as proliferation, differentiation, apoptosis and

stress response. However, the function of a miRNA depends on mRNA target. A single miRNA may target multiple genes and vice versa, one mRNA may be regulated by more than one miRNA.

1.3.3 miRNA and cancer

A study conducted in Croce’s lab revealed the involvement of miRNAs in cancer. In particular, miR-15a and miR-16-1 are codified by a region frequently deleted in chronic myeloid leukemia (CML). These two miRNAs act as oncosuppessor and loss of that genomic portion may be the initial event for leukemogenesis in CML (Croce, 2009). After this observation, the same group began to map all the known genes encoding for miRNA. These genes are usually located in chromosomic region which undergo in rearrangements in several tumors. They suggested that miRNAs can act both as oncogene and oncosuppressor. Chromosomic regions containing oncosuppressive miRNA are located in fragile sites where deletions and mutations occurs more frequently. miRNAs with oncogenic features map in region prone to amplification. These evidences highlight that miRNAs expression alterations have a key role in neoplastic transformation (Calin, 2003).

Several mechanisms control miRNAs expression and they are aberrantly modulated in cancer. The first mechanism studied were chromosomic rearrangement. Then, miRNA genes may be interested by sequence variations, like SNP. If a SNP occurs in the seed sequence, the spectrum of target genes changes, favoriting oncogenesis. However, no tumor-specific mutations have been reported yet (Iorio, 2012). Like genes encoding for protein, miRNA genes may be involved in epigenetic change. miRNA loci are associated to CpG islands, thus changes in DNA methylation are recorded. Genes of oncosuppressive miRNA present hypermethylated promoter region, instead oncomiR genes are hypomethylated. Also, histone acetylation represents an epigenetic mechanism which is deregulated in cancer. Reduction in histone acetylation decreases anti-oncogenic miRNA expression.

However, miRNAs regulate expression of components of the epigenetic machinery, resulting in an intricated scenario of feedback regulation. Deregulation of miRNA expression could reflect an altered transcriptional activity. Also, miRNAs could be modulated as result of defect in their biogenesis. Dicer and Drosha have oncosuppressive features and their silencing induces tumorigenesis *in vivo*. Alteration on miRNA target genes have been observed such as variants on target sequences and secondary structures formation. These secondary structures can impair the formation of miRNA-mRNA annealing. By this mechanism the regulation of miRNAs towards their targets is hampered (Iorio, 2012).

Usually, miRNAs are repressed in tumoral tissue respect to the normal counterpart, indicating a general loss of differentiation. miRNA deregulation is associated to neoplastic transformation. Indeed, miRNAs influences all six hallmarks of malignant cells: a) sustain proliferative signaling (let-7 family); b) evasion growth suppressors (miR-17/19 cluster); c) resistance to apoptosis (miR-34a); d) infinitive replicative potential (miR-372/373 cluster); e) angiogenesis (miR-210); f) invasion and metastasis (miR-10b) (Di Leva, 2014).

miRNA expression is strictly tissue- or cellular-specific, so each miRNA could act as oncomiR or oncosuppressor miRNA, depending on the cellular context.

1.3.4 miRNA as biomarkers

miRNA expression profiling studies are based on alteration of miRNA in tumoral tissue respect normal ones. Several techniques have been developed for evaluating miRNA expression. Most used are Microarray and Next Generation Sequencing, qPCR and in-situ hybridization, depending on the aim of the study (expression of all miRNAs or one specific miRNA). First studies of miRNAs profiling highlighted how these profiles could classify different tumoral types. Lots of studies demonstrated how several groups of miRNAs are mainly deregulated in specific tumors and other are associated to diagnosis, staging, progression, prognosis and chemotherapy response (Iorio, 2011; Di Leva, 2013). Cancer shows a specific miRNA signature, called miRNome or miRNA fingerprinting, which characterizes malignant state and defines some clinic-pathological features. miRNA may have prognostic and therapeutic potential and an important role in tumorigenesis (Di Leva, 2014).

Biomarkers are biological indicators of pathological state and they are useful tool for defining tumoral subtypes or evaluating therapy efficacy. miRNAs could be perfect biomarkers in clinical practice thanks to: their stability in formalin fixed samples, relative ease quantification, potential in discriminating pathological state and association with clinico-pathological characteristics (Hauptam, 2013). Discovery that miRNAs are present in biological fluid, such as serum, saliva, urine, blood and breast milk, leads to an increased number of studies for identifying miRNA as non-invasive biomarkers predictive of both disease progression and therapeutic responses (Shen, 2013).

2. AIM OF THE PROJECT

Osteosarcoma is the most frequent primary tumor of the bone, characterized by an aggressive and metastatic potential. Few improvements were achieved in term of therapies in the last two decades. Understanding mechanisms underlying chemosensitivity of tumor cells and the discovery of new prognostic factors could improve patients' outcome. On the other hand, a relationship between tumor and immune system has been extensively demonstrated. The interplay between tumors and their immunologic microenvironment is complex and difficult to understand, but a greater comprehension of this interconnection is important for the development of novel prognostic markers and improve therapeutic strategies. Only sporadic and controversial information are available for the immune cells infiltrating osteosarcoma. It has been demonstrated that CD99 shows oncosuppressive features in osteosarcoma. In fact, it's expressed at low level, induces cell adhesion to ECM and inhibits migratory and anchorage-independent growth abilities *in vitro*, while *in vivo* suppresses tumorigenic and metastatic potential. Furthermore, this protein plays a key role in modulating several functions of immune cells, such as proliferation and activation of lymphocytes, regulation of MHC class I molecule transport, T-cell migration and diapedesis of monocytes across endothelium. In this study, expression of CD99 has been evaluated by IHC in 100 osteosarcoma biopsies, discovering the potential of CD99 as a predictive biomarker of ifosfamide response. The importance of CD99 in ifosfamide sensitivity has been studied in osteosarcoma cell lines. For the same tumor specimens, expression of immune infiltration markers and their correlation with CD99 has been considered. For understanding the regulation of CD99 in osteosarcoma, miRNA mediated post-transcriptional regulation has been examined. Prognostic relevance of miRNAs emerged in the last years. Thanks to tissue- and cell- specificity, it is possible to identify a miRNA signature able to discriminate patients with better prognosis. A small RNA sequencing pilot study has been conducted on 14 OS samples, with the aim to identify a miRNome able to distinguish at diagnosis patients with high probability to relapse. This signature has been validated by qPCR in a larger OS cohort.

3. MATHERIALS AND METHODS

Immunohistochemistry

Osteosarcoma specimens were incorporated in 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 osteosarcoma biopsies slides and then sampling tissue from the corresponding paraffin blocks. A tissue microarray instrument (Beecher Instruments Inc, Sun Prairie, WI, USA) was used for TMA assembly. From TMA blocks, 3-μm-thick sections were immunostained using mouse anti-CD99. Percentage of positive cells and cytoplasmic staining intensity were scored in semiquantitative manner. Data of immunostaining for CD3, CD8, CD20, CD68, PD-1, PDL-1, FOXP3 were already available.

Cell lines

Cell lines U-2 OS, SaOS-2 and HEK293 were obtained from American Type Colture Collection (Rockville, MD, USA); IOR/OS9, IOR/OS10, IOR/OS15, SARG, OS-PDX#1-C4, OS-PDX#19-C2 were stabilized and characterized at CRS Development of Molecular Therapies, Laboratory of Experimental Oncology, Rizzoli Orthopaedic Institute (Bologna, IT). U-2 OS stably transfected for overexpression of CD99 were previously described in Manara *et al* (Manara, 2006). Each cell line has been tested for mycoplasma contamination (MycoAlert™ mycoplasma detection kit, Lonza, Verviers, Belgium) and authenticated by STR PCR analysis. Cells were cultured in IMDM (Lonza), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% inactivated fetal bovine serum (FBS) (Lonza).

MTT assay

To assess drug sensitivity, MTT assay (Trevigen Inc, Gaithersburg, MD, USA) was used according to manufacturer’s instructions. Cells were plated into 96 well-plates (2500 cells/well). After 24 hours, various concentrations of doxorubicin (DXR 0.3 -1000 ng/ml) (Pfizer, New York City, NY, USA), methotrexate (MTX 1 - 300 nM) (Teva Italia srl, Milano, IT), cisplatin (CDP 0.3 – 3 μg/ml) (Teva Italia srl), 4-hydroperoxyifosfamide (4-HOO IFO 0.3 - 100 μM) (Niomech

– IIT GmbH, Bielefield, Germany) were added and cells exposed to these drugs for up to 96 hours.

Flow cytometry analysis

The expression of ABC transporters was analyzed by indirect immunofluorescence and flow cytometry analysis (FACS Calibur, Becton Dickinson, Milan, IT). Cells were fixed in 4% PFA and washed with PBS-BSA1%-Tween20 0.5% (PAT) and permeabilized with 0.15% Triton X in PBS. Then they have been resuspended in anti-MRP1 1:20 + Goat anti-mouse IgG (H +L) FITC 1:100 (Thermo Fisher Scientific Inc, Waltham, MA, USA); anti-MRP4 1:20 + Goat anti-rat IgG (H +L) FITC 1:40 (KPL); anti-ABCG2 1:20 + Goat anti-mouse IgG (H +L) FITC 1:100 (Thermo Fisher Scientific Inc).

For cell cycle analysis, cell cultures were incubated with 10 μM bromodeoxyuridine (BrdUrd) (Sigma-Aldrich, Milan, IT) for 1 h in CO_2 atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 min. After DNA denaturation with 2 N HCl for 30 min at room temperature, cells were washed with 0.1 M $Na_2B_4O_7$, pH 8.5, processed for indirect immunofluorescence staining, using α-BrdUrd (Becton Dickinson) diluted 1:4 as a primary MAb, stained with 20 μg/ml propidium iodide and analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

RNA extraction and Real-Time PCR

Total RNA from tumor samples was isolated from snap-frozen tissue material using Trizol LS Reagent (Thermo Fisher Scientific Inc). To check whether extracted RNA was representative of OS cells, the tissue was morphologically analyzed after hematoxylin–eosin staining. Oligo dT primers (Thermo Fisher Scientific Inc) 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 (Thermo Fisher Scientific Inc). Expression of several genes belonging to immunological pathway were performed through TaqMan™ Array Human Immune Panel. Expression levels of target genes were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

CD99 expression was performed using Predesigned TaqMan probes (Hs00908458_m1) (Thermo Fisher Scientific); expression data were normalized to GAPDH levels.

qRT-PCR analysis of miRNAs has been carried out using TaqMan® MicroRNA Assays and TaqMan® Universal PCR Master Mix, no AmpErase® UNG (Thermo Fisher Scientific)

according to manufacturer's instructions. RNU6b was used as reference gene for expression data normalization.

The relative quantification analysis was performed on the basis of the 2-ΔCT method, in order to compare two samples groups (NED vs REL).

miRNA-mRNA target prediction.

miRNA target prediction of CD99-3'UTR was performed through the webtool MirWalk v.2 [6], considering targets predicted with a consensus of at least 6 tools.

Luciferase assay

To generate CD99 luciferase reporter constructs, the 3′UTR of the gene was cloned downstream of the luciferase-coding sequence in the psiCHECK-2 at the XhoI and NotI restriction sites (Promega, Madison, WI, USA). Mutations were introduced into the miRNA-binding sites by using the QuikChange Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). All the transfection studies were performed with 500 ng of each plasmid and 100nM of pre-miRNA. Luminescence signal were detected through GloMax Multi Detection system (Promega) according to manufacturer's instructions.

Western blotting

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% sodium deoxycholate, 1mM NaF) upon addition of proteases inhibitors (1:100): aprotinin (10μg/ml), leupeptin (0.1mM), PMSF (1mM), sodium orthovanadate (0.2mM). After 30 minutes incubation, lysates were centrifuged and proteins from solution fraction were collected. Protein concentration was calculated 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 denaturating conditions and transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Milan, IT). Red Ponceau S (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 then incubated overnight with the following primary antibodies: anti-actin, anti-CD99, anti-ROCK2. Membranes were then incubated with secondary anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, UK) and revealed by ECL Western blotting detection reagents (GE Healthcare).

In Vitro Migration Scratch.

U-2 OS osteosarcoma cells were transfected with antagomiR-330 (Thermo Fisher Scientific Inc). Twenty-four hours after transfection, a cell-scratch spatula was used to make a scratch in the cell monolayer, after which the cell monolayers were rinsed and further incubated. Pictures of the scratches were taken by using a digital camera system coupled to a microscope (Nikon 90i Eclipse) (Nikon Corporation, Tokyo, Japan)

Statistics

Differences among means where analyzed using two-sided Student's t test (Graphpad Prism). IC50 values were determined using CalcuSyn software (Biosoft). Correlations analysis were performed using Spearman correlation test. Kaplan-Meier proportional risk log rank test was applied for survival curves. Event-free survival (EFS) and overall survival (OVS) were considered individually from the date of diagnosis to the date of event. Univariate predictors of outcome were entered a Cox proportional hazard model using stepwise selection to identify independent predictors of prognosis, considering the 95% CI (IBM SPSS).

Small RNA sequencing

21 samples were submitted for small RNA sequencing through Illumina Platform. Integrity of RNA was evaluated by 2100 Bioanalyzer (Agilent Technologies) and samples with RIN > 7 were analyzed. Library preparation and sequencing were performed according to manufacturer's instruction (Illumina, San Diego, CA, USA)

sRNAseq Analysis

sRNAseq sample files were firstly trimmed of Illumina standard 3' adaptor sequences via the tool Cutadapt. Trimmed samples were then analysed for miRNA profiling leveraging on miRBase version 20 annotation database and by employing the pipeline miARma-Seq, which included the tools: Bowtie v1 to align trimmed reads against the human genome (GRCh37), featureCounts for read count summarizing, and the Bioconductor package EdgeR for differential expression analysis. As recommended, reads were normalized using the TMM method (trimmed mean of M-values) and the exact tests to calculate differences between two groups of negative-binomial counts was performed to gather the log2 fold change, as well as the Benjamini and Hochberg false discovery rate (FDR) for each miRNA. Subsequently, expressed miRNAs were retained if their average expression counts were >100 in at least one condition. From these, those with an absolute log2 fold change (log2FC) value >1 were selected for subsequent analyses.

Gene expression Analysis

Gene expression profile was performed by GeneChip® Human Transcriptome Array 2.0 (HTA 2.0 - Affymetrix) for the same 21 samples used for smallRNA seq experiment. Gene level normalization was carried out by the Affymetrix® Expression Console™ Software, while differential expression (DE) analysis was performed employing the Transcriptome Analysis Console (TAC) 3.0 Software. The One-Way between-subject ANOVA algorithm was used on normalized data to calculate statistical significances of pairwise comparisons.

miRNA-gene Anticorrelation Study

Pearson correlation coefficient was calculated on miRNA/gene pairs for miRNAs hsa-miR-130b-5p, hsa-miR-139-5p and hsa-miR-99b-3p, and all genes with an official gene symbol reported in the HTA 2.0 Affymetrix gene expression analysis above. All pairs with Pearson correlation coefficient < 0 and p-value < 0.05 were selected for further analyses.

Functional Enrichment Analysis

For each miRNA mentioned above, the set of predicted targets was intersected with the set of anti-correlated genes and the resulting set was used as input for a functional enrichment analysis by Ingenuity® Pathway Analysis (IPA®) software. Settings used included experimentally observed data for the human species.

4. RESULTS

4.1 CD99 as predictive biomarker in Osteosarcoma

4.1.1 Evaluation of prognostic potential of CD99 in OS samples

CD99 was evaluated by immunohistochemistry in 100 primary samples of osteosarcoma.

These patients were enrolled into ISG/OS-1, a multicenter randomized trial comparing efficacy and toxicity of two chemotherapy regimens, both based on MTX, CDP, ADM, and IFO and with the same cumulative doses but administered according to different schemes. The protocols had a different dose-intensity and different modality of use of IFO. In arm A, IFO was given only postoperatively and in patients with poor histologic response to primary chemotherapy with MTX, CDP, and ADM. In arm B, IFO was delivered in all patients and in the primary phase of chemotherapy. All patients enrolled in this study were affected by high grade OS of extremities, non-metastatic at diagnosis, younger than 40 years old.

Median follow-up of the 100 OS patients was 113 months (range 9–167 months). Fifty-three patients (53%) remained continuously free of disease and 47 (47%) relapsed; sixty-seven patients were alive and thirty-three dead. The clinical-pathological features of the cohort and the correlation with event-free survival and overall-survival are summarized in the Table 1.

Characteristics	N.	%	EFS	OVS
Gender				
Female	37	37	0.139	0.096
Male	63	63		
Age				
< 14 years	40	40	0.214	0.147
≥ 14 years	60	60		
Histotype				
Chondroblastic	10	10	0.162	0.522
Fibroblastic	10	10		
Osteoblastic	68	68		
Telangectatic	9	9		
ND	3	3		
Location				
Femur	47	47	0.164	0.743
Fibula	7	7		
Humerus	18	18		
Tibia	28	28		
Necrosis				
Good	52	52	<0.001	0.002
Poor	47	47		
ND	1	1		
Alkaline phosphatase				
High	43	43	0.016	0.035
Normal	54	57		
N.D.	3	3		
LDH				
High	25	25	0.005	0.030
Normal	66	68		
ND	9	9		

Table1. Clinico-pathological features of OS cohort and association with survival

The patients have been stratified as negative and positive for the expression of CD99: 53 patients showed a positive expression for the molecule, 45 were barely negative for CD99; for 2 patients, data about immunostaining are not available.

According to the tumor-suppressor role of CD99 in OS, a significant and positive association between CD99 expression and EFS was found (p-value= 0.036) when considering the entire cohort, as shown in figure 1.

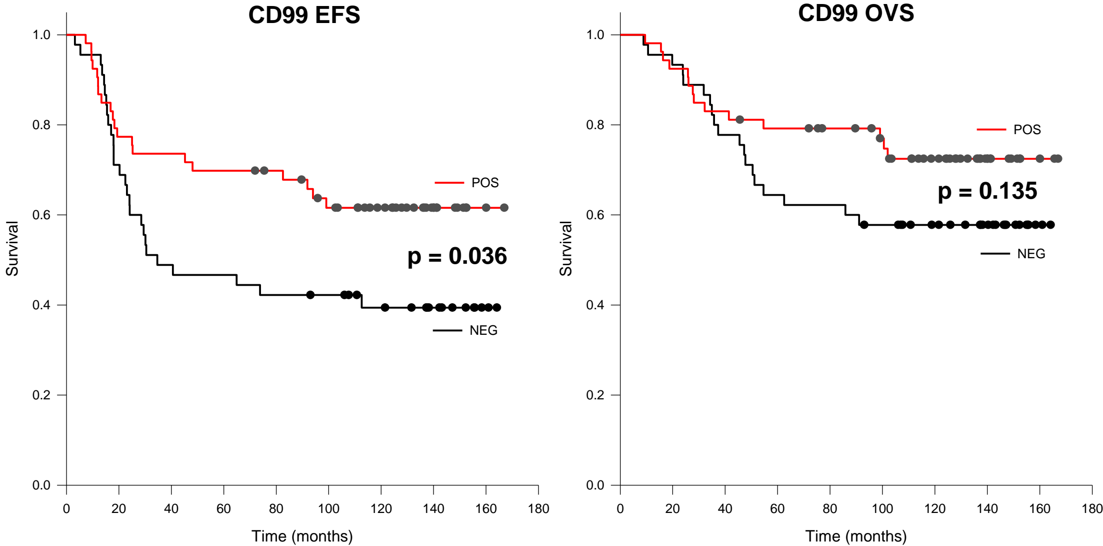

Figure 1. Kaplan Meier Survival Curves based on CD99 expression in OS

The importance of CD99 as prognostic marker was confirmed by multivariate analysis (Cox's regression test). Results of Cox's regression test are summarized in Table 2.

Variables associated with worse EFS in the entire cohort	RR	95% CI	p-value
NECROSIS: poor	3.401	1.486 to 7.576	0.004
Alkaline phosphatase: high level	2.247	1.011 to 4.995	0.047
Lattate dehydrogenase: high level	2.870	1.284 to 6.415	0.010
CD99: negative expression	2.164	1.016 to 4.609	0.045

Table 2. Multivariate analyses for EFS in the entire cohort

Then, the univariate analysis was conducted also in the two different arms of randomized study separately. When only the samples derive from the arm A were considered, CD99 lost its association with a better prognosis (EFS p value= 0.670, OVS p-value= 0.318). However, when we focused our attention on arm B, CD99 was associated with a better prognosis both in EFS and OVS, with p=0.001 and p=0.003 (Figure 3).

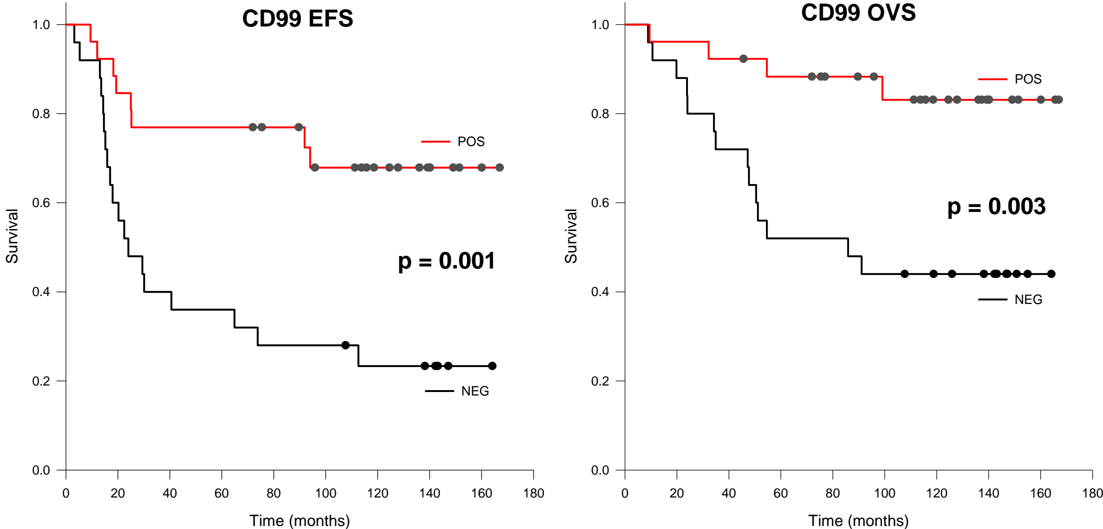

Figure 3. Kaplan Meier Survival Curves based on CD99 expression for patients belonging to arm B

Cox regression test confirmed the increased risk of relapsing and poor prognosis, as shown in the tables.

Variables associated with worse EFS in arm B	RR	95% CI	p-value
CD99: negative expression	3.530	1.440 to 8.657	0.006

Variables associated with worse OVS in arm B	RR	95% CI	p-value
CD99: negative expression	5.212	1.582 to 17.173	0.007

Table 3 and 4. Multivariate analyses for EFS (3) and OVS (4) in arm B

These associations are probably due to an enhanced sensitivity to the ifosfamide induced by CD99.

4.1.2 CD99 is implicated in the sensitivity of ifosfamide

To test the hypothesis that CD99 mediates the sensitivity to ifosfamide, the difference of efficacy in OS cell was analyzed, correlating the IC_{50} to ifosfamide to the expression of CD99.

First, two cell lines derived from xenografts was tested: one positive for CD99 (PDX-OS#19-C2), the other barely negative (PDX-OS#2-C4). It was found that the cell line positive for CD99 was more sensitive to 4-HOO IFO (the active compound of ifosfamide used for in vitro studies). This difference is statistically significant (Student's t test p-value = 0.033).

Data from CD99 expression analyses by FACS and IC_{50} doses for each cell lines analyzed were reported in table 5.

Cell line	% of positive cells	Log Mean	IC_{50} 4-HOO-IFO
PDX-OS#1-C4	26.5	6.1	12.5 ± 1.7 µg/mL
PDX-OS#19-C2	77.8	23.3	2.8 ± 0.6 µg/mL

Table 5. CD99 expression analyses and IC50 doses for two cell lines stabilized from PDX

Then, the sensitivity of OS cells with a forced expression of CD99 was tested against the four drugs of the clinical trial. As shown in the graphs below, expression of CD99 did not affect the sensitivity to doxorubicin, methotrexate and cisplatin, where there were no changes in the IC_{50} doses between cells with forced expression of CD99 and the parental ones. However, IC_{50} for ifosfamide was significantly reduced in U-2/CD99wt 57 (IC_{50} = 7.3 ± 2.5 µg/mL, Student's t test p-value < 0.001) and in U-2/CD99wt 136 (IC_{50}=12.6 ± 2.5µg/mL, Student's t test p-value = 0.04) compared to parental cell line (IC_{50}= 21.9 ± 0.9 µg/mL). Therefore, CD99 can enhance the sensitivity to ifosfamide, but has no effect in increasing the sensitivity to other drugs, as shown in figure 4.

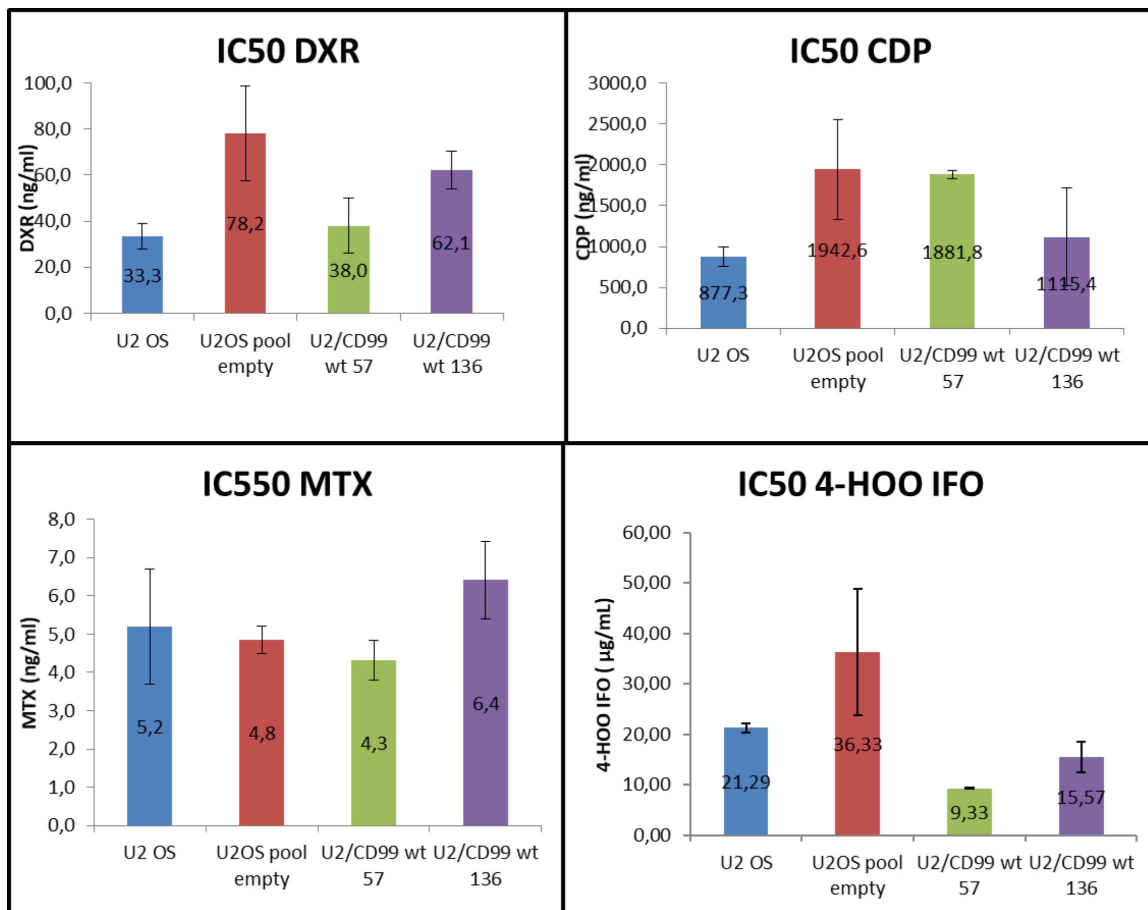

Figure 4. IC50 doses for DXR, CDP, MTX, 4-HOO IFO of OS cell line stably transfected for overexpression of CD99; it's represented the mean of at least three independent experiment

To better comprehend why CD99 can promote the sensitivity to ifosfamide, cell cycle and apoptosis have been evaluated. After 48 hours of 10 µg/mL treatment, there was an increase in the number of cells in S phase when CD99 was overexpressed (Figure 5). This data is consistent to the mechanism of action of IFO.

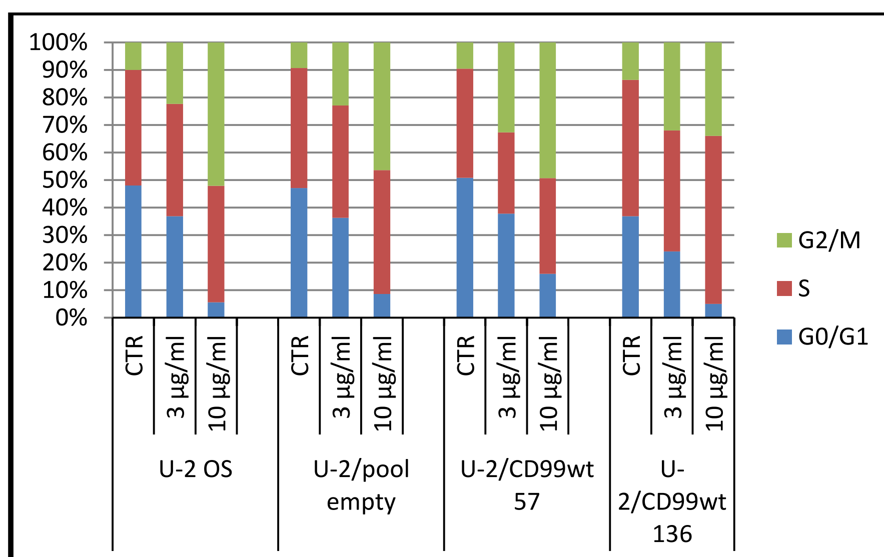

Figure 5. Cell cycle analyses after 48 hours of treatment with two doses of 4-HOO IFO (3 and 10 µg/mL)

In addition, an increased proportion of apoptotic cells was observed in cells overexpressing CD99, especially with the treatment of 10 µg/mL 4-HOO IFO respect the parental ones in the same treatment condition, as shown in figure 6.

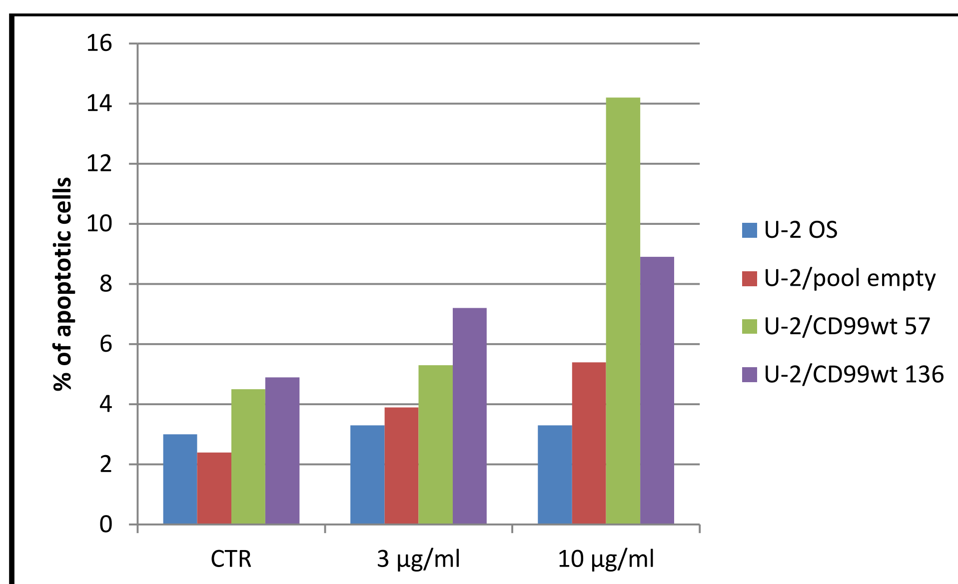

Figure 6. Percentage of apoptotic cells analyzed as hypodiploid peak

To try to explain why and how CD99 can modulate the sensitivity to ifosfamide, some mechanisms involved in resistance, like active uptake and reflux, were considered. Thus, the basal expression of MRP1, MRP4 and ABCG2 was analyzed by FACS in transfected cells.

	MRP1		ABCG2		MRP4	
	% positive cells	LOG MEAN	% positive cells	LOG MEAN	% positive cells	LOG MEAN
U-2 OS	99.2	14.7	77.4	7.8	53.6	7.6
U-2/pool empty	98.9	19.5	36.2	3.7	85.9	12.9
U-2/CD99wt 57	99.5	23.1	72.8	7.4	95.2	19
U-2/CD99wt 136	88.7	16.3	62.6	9.8	94.5	16.4

Table 6. FACS analyses of MRP1, MRP4 and ABCG2 in U2-OS and CD99 overexpressing clones

As shown in the previous table, no differences that could explain the difference in sensitivity to ifosfamide were observed.

4.1.3 CD99 and immune infiltrates in osteosarcoma

The results presented so far showed that the expression of CD99 can modify the sensitivity of osteosarcoma cells to ifosfamide. Considering the relevance of CD99 in lots of processes of immune system, the prognostic role of CD99 may also reflects its ability to communicate with immunological cells.

For the same cohort analyzed for CD99 expression data were available in IHC for some markers of the immune infiltrates. Frequencies and association with prognosis are reported for each immunological marker evaluated in table 7.

	EXPRESSION	N	%	EFS	OVS
CD3	None	10	11.0	0.280	0.204
	Focal	62	68.1		
	Moderate	19	28.9		
CD8	None	13	14.4	0.001	0.013
	Focal	59	65.6		
	Moderate	18	20.0		
CD20	None	32	69.7	0.309	0.366
	Focal	24	26.9		
	Moderate	3	3.4		
CD68	None	1	1.2	0.042	0.097
	Focal	52	60.4		
	Moderate	28	32.6		
	Severe	5	5.8		
FOXP3	None	58	66.7	0.027	0.216
	Focal	28	32.1		
	Moderate	1	1.2		
PD1	None	71	78.9	0.487	0.633
	Focal	16	17.8		
	Moderate	3	3.3		
PDL1	None	80	87.0	0.671	0.178
	Focal	12	13.0		

Table 7. List of immune infiltrates markers evaluated in OS cohort and correlation with survival

As expected, the prognosis was better in term of both EFS and OVS when there was a prevalence of CD8+ T cells. Successively, a Spearman correlation test was performed to indagate correlations between the immune infiltrates markers and CD99. Results are summarized in table 8.

Spearman Correlation		**CD8**	**PD1**	**FOXP3**	**CD20**	**CD68**	**PDL1**	**CD99**
CD3	Rho	0.875	0.364	0.382	0.387	0.387	0.406	0.178
	(p-value)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.093)
CD8	Rho		0.341	0.325	0.451	0.416	0.417	0.236
	(p-value)		(0.001)	(0.002)	(<0.001)	(<0.001)	(<0.001)	(0.026)
PD1	Rho			0.400	0.240	0.470	0.210	0.280
	(p-value)			(<0.001)	(0.030)	(<0.001)	(0.048)	(0.009)
FOXP3	Rho				0.217	0.335	0.223	0.151
	(p-value)				(0.045)	(0.012)	(0.038)	(0.165)
CD20	Rho					0.443	0.265	0.125
	(p-value)					(<0.001)	(0.012)	(0.245)
CD68	Rho						0.138	0.247
	(p-value)						(0.205)	(0.023)
PDL1	Rho							-0.007
	(p-value)							(0.946)

Table 8. Summary of Spearman correlation between markes analyzed in the OS cohort

All significant correlations with CD99 are weak due to the technique used for the expression analysis.

Univariate analyses were conducted in some subgroups like CD8 and CD99 positive patients versus CD8 and CD99 negative, CD68 and CD99 positive versus CD68 and CD99 negative. These tests were carried out to understand if the association between those markers could improve the prognosis of patients and if there is any difference considering the entire cohort despite only arm B. The survival analysis in the whole cohort shows that patients positive for both CD8 and CD99 have a better prognosis in terms of EFS (p-value < 0.001) and OVS (p-value = 0.016). Similar results were obtained regarding positive expression of CD99 and CD68 (EFS p-value=0.023; OVS p-value= 0.033). When only the patients who received ifosfamide were considered, the p-value obtained enhanced the role of CD99 in the sensitivity of ifosfamide. As a matter of fact, the significance of the univariate analysis increases, as shown in figure 7.

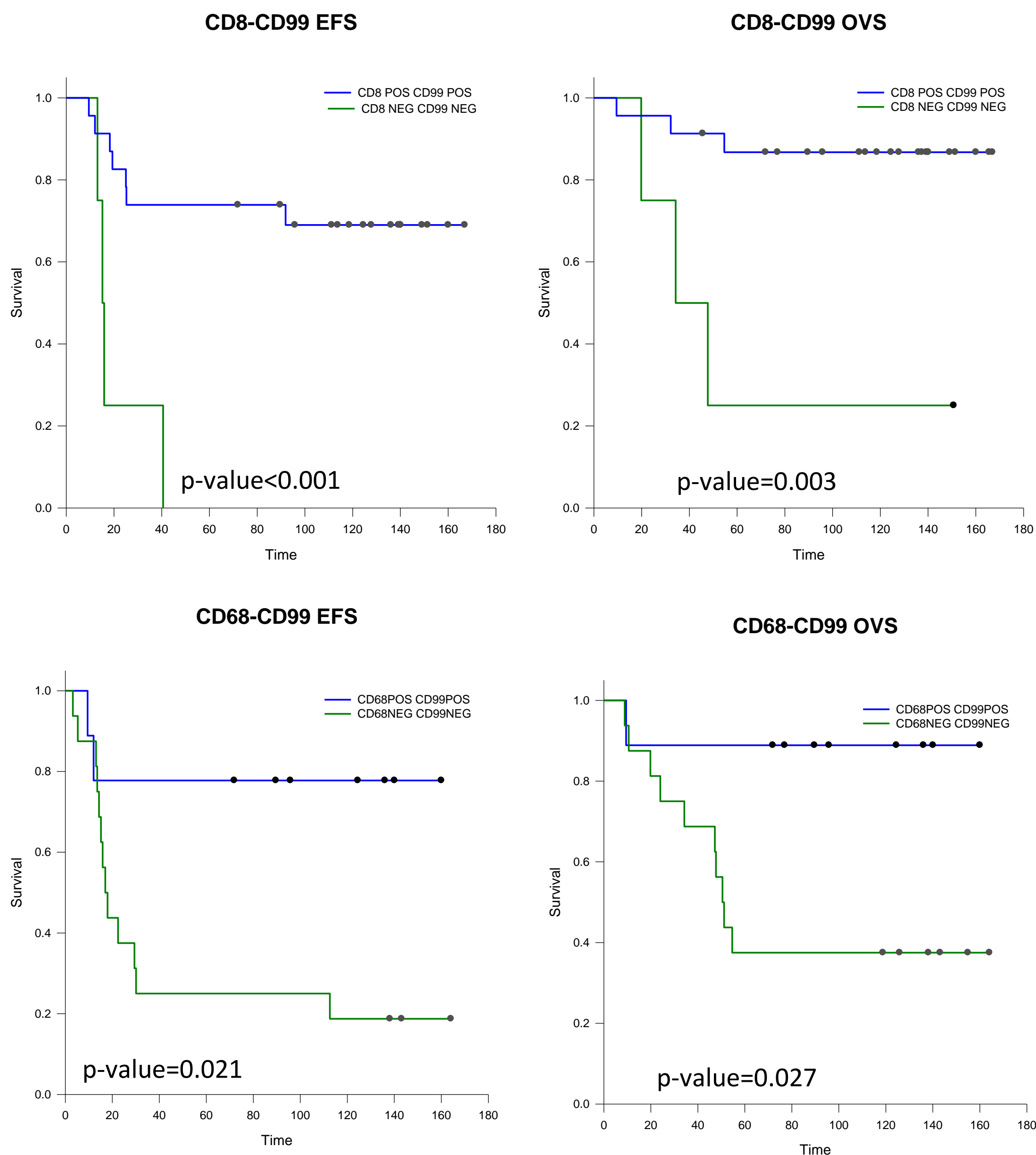

Figure 7. Kaplan Meier Survival Curves based on CD8 and CD99 positive patients versus CD8 and CD99 negative, CD68 and CD99 positive versus CD68 and CD99 negative in arm B

The simultaneous presence of CD8 and CD99 (or CD68 and CD99) predicts a better prognosis in the entire cohort (data not shown); the significance of these correlations is improved when the analyses were performed considering the B arm. These data suggest that CD99 is important for a greater response to ifosfamide also due to its possible role in modifying the composition of immune infiltration of the tumor.

To give more strength to these correlation between CD99 and CD8 or CD68, a second independent cohort of OS was analyzed by qPCR. The cohort is composed of 48 patients affected by high grade OS of extremities, non-metastatic at diagnosis, younger than 40 years old.

All patients received neoadjuvant treatment. Half of these patients belongs to NED groups, the others to REL. Expression of several markers proper of immunological systems was analyzed through TaqMan™ Array Human Immune Panel. All these analyses were performed using GAPDH as reference gene. It was found that CD99 correlates with CD8A (Rho=0.508 p-value=0.006) and CD68 (Rho=0.381 p-value=0.045).

Altogether, these results indicate that the expression of CD99 correlates with CD8 and CD68 at both mRNA and protein level.

4.2 Post-transcriptional regulation of CD99

In order to better comprehend the role of CD99 in human high-grade OS, the attention was focused on the expression's regulation of CD99 mediated by miRNA.

4.2.1 *In silico* study

The first step for investigating miRNA regulation is to study the 3' UTR of gene of interest. This is the most prominent part of the *in silico* approach of this kind of research. There are lots of software that can be used for prediction analyses of miRNA:mRNA interaction. Usually the prediction analysis is the combination of results obtained by at least four different software. In this case seven software were interrogated. Successively, there is the need to study the predicted miRNAs on miRNA database (like miRbase) especially for understanding their role (if the protein of interest has a tumor suppressor role, is better to choose an oncomiRNA), knowing the relative expression of the 2 isoforms (5p or 3p) and finally the involvement of these miRNAs in any disease.

The chosen candidates for targeting CD99 are hsa-miR-23a-3p, hsa-miR-330-3p, hsa-miR-27a-5p, hsa-miR190b. The sequence of CD99 and the seed sequence for each miRNA of interest are represented in figure 8.

CD99 - NM_002414 - 3'UTR

1	AAGATTGTCGGCAGAAACAGCCCAGGCGTTGGCAGCAGGGTTAGAACAGC	50
51	TGCCTGAGGCTCCTCCCTGAAGGACACCTGCCTGAGAGCAGAGATGGAGG	100
101	CCTTCTGTTCACGGCGGATTCTTTGTTTTAATCTTGCGATGTGCTTTGCT	150
151	TGTTGCTGGGCGGATGATGTTTACTAACGATGAATTTTACATCCAAAGGG	200
201	GGATAGGCACTTGGACCCCCATTCTCCAAGGCCCGGGGGGGCGGTTTCCC	250
251	ATGGGATGTGAAAGGCTGGCCATTATTAAGTCCCTGTAACTCAAATGTCA	300
301	ACCCCACCGAGGCACCCCCCCGTCCCCCAGAATCTTGGCTGTTTACAAAT	350
351	CACGTGTCCATCGAGCACGTCTGAAACCCCTGGTAGCCCCGACTTCTTTT	400
401	TAATTAAAATAAGGTAAGCCCTTCAATTTGTTTCTTCAATATTTCTTTCA	450
451	TTTGTAGGGATATTTGTTTTTCATATCAGACTAATAAAAAGAAATTAGAA	500
501	ACCAA	505

hsa-miR-23a-3p - miRiam + RNAhybrid
hsa-miR-330-3p - RNAhybrid
hsa-miR-190b - TargetScan 7.0
hsa-miR-27a-5p - miRiam + RNAhybrid

Figure 8. 3'UTR sequence of CD99 and predicted seed sequence for has-miR23a-3p(light blue), hsa-miR-330-3p (green), hsa-miR-190b (yellow), has-miR-271-5p (purple)

4.2.2 *In vitro* analysis

The following step is included in the *in vitro* part and consists of validation of targeting through luciferase assay, by WB and qPCR in the cellular model of interest and some functional studies depending on the role of the protein and miRNA.

First, the 3'UTR of CD99 were cloned into a commercial plasmid, the psi-check2 plasmid by Promega. This plasmid carries two luciferases, firefly and renilla. CD99-3'UTR is cloned fused with the 3' extremity of renilla luciferase gene. Firefly luciferase is constituvely expressed and its luminescence signal is used as calibrator. The luciferase assay consists of a double transient transfection of HEK293 cells with the plasmid and the pre-miRNA of interest. If the identified miRNA does not target CD99, the luciferase is expressed and there is a luminescence signal. If the miRNA targets the 3'UTR there is no luminescence signal due to the miRNA-mediated downregulation.

In the figure below there is a schematic representation of the test and how it works.

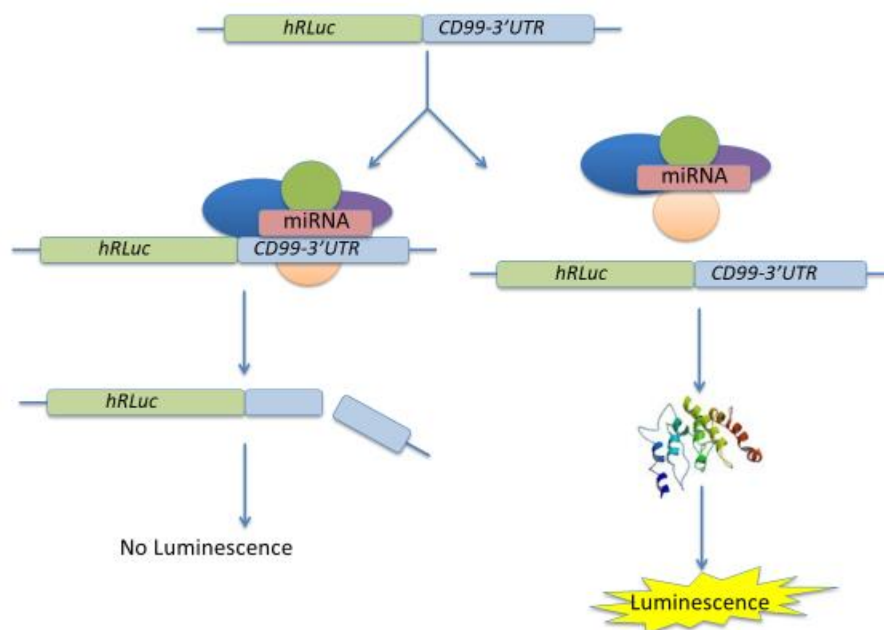

Figure 9. Schematic representation of luciferase assay

The data obtained with the luciferase assay are reported in figure 10. All differences in activity compared with a negative control were significant, except for miR-190.

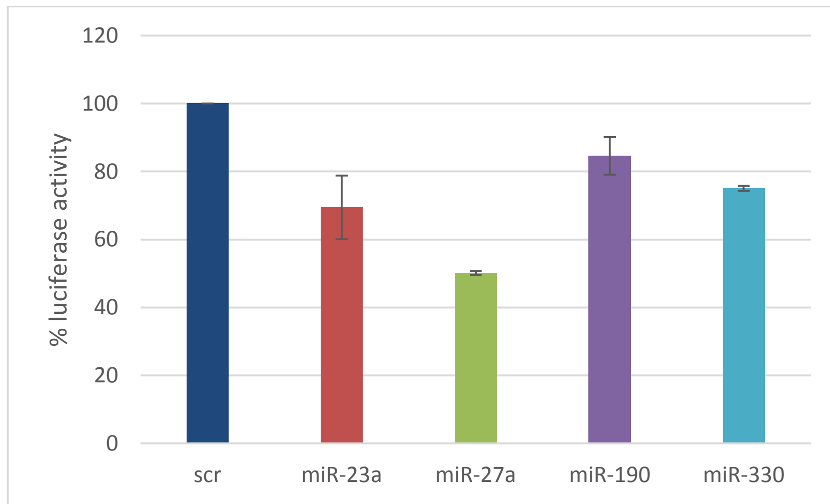

Figure 10. Luciferase assay results

For each miRNA that effectively decreases the luminescence, a mutagenic plasmid was created in which the seed sequence was deleted. For miRNA having more than one seed sequence was performed a site mutagenesis for each site and a plasmid containing all the mutated sequences together. Results are shown in figure 11.

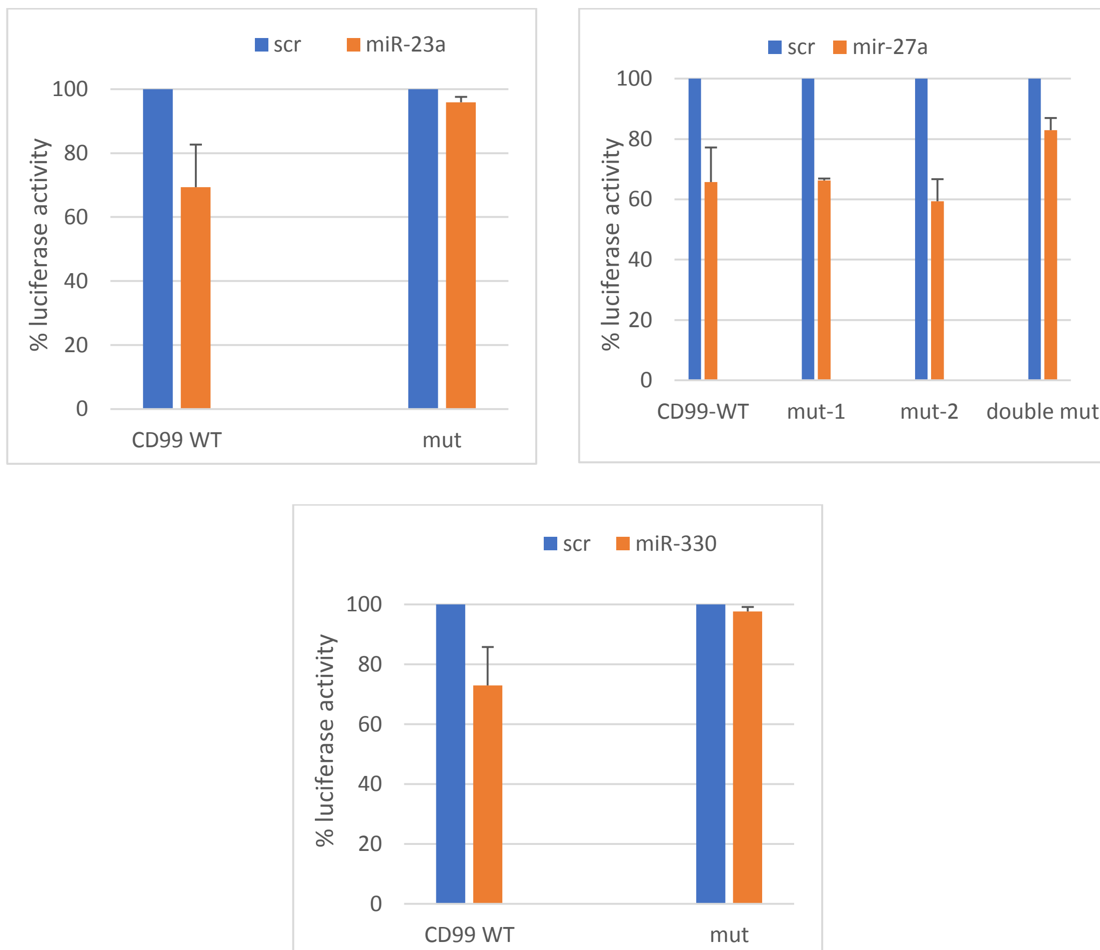

Figure 11. Luciferase assay data obtained with site-specific mutagenized plasmid

As shown, if the seed sequence was deleted, miRNA was not able to bind the 3'UTR and there was no change in the luminescence signal. These data proved the specificity of the binding of the predicted miRNAs to CD99.

Furthermore, it was necessary to confirm the targeting in the model of interest, both through Real Time PCR and Western Blotting. After 48 hours of transfection with pre-miRNAs in OS cell line, the expression level of CD99 was evaluated both as mRNA and protein.

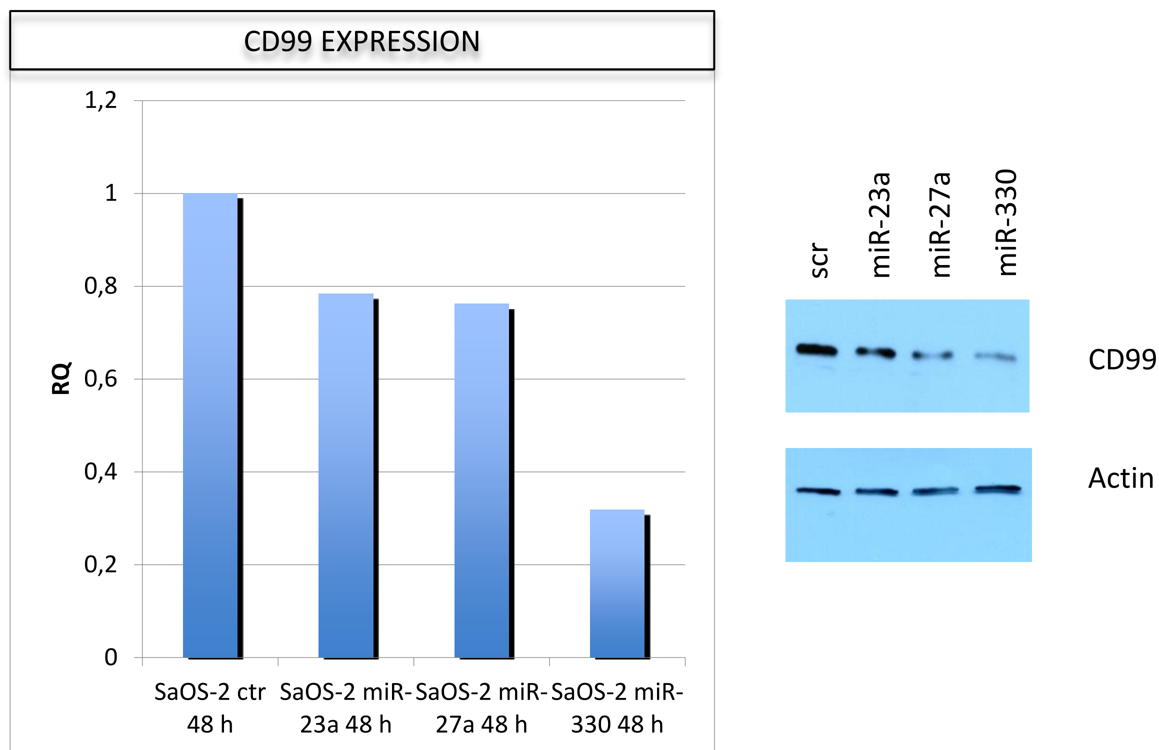

Figure 12. CD99 targeting confirmation by qPCR and WB

After the treatment with the pre-miRNAs there was a decrease of the target molecule; based on these results miR-330-3p was chosen for further studies.

First, miR-330-3p expression was evaluated in a panel of OS cell lines. A strong anticorrelation (Spearman correlation test, rho= -0.930, p-value= 0.017) was observed between the expression of miR-330 and CD99, corroborating the targeting.

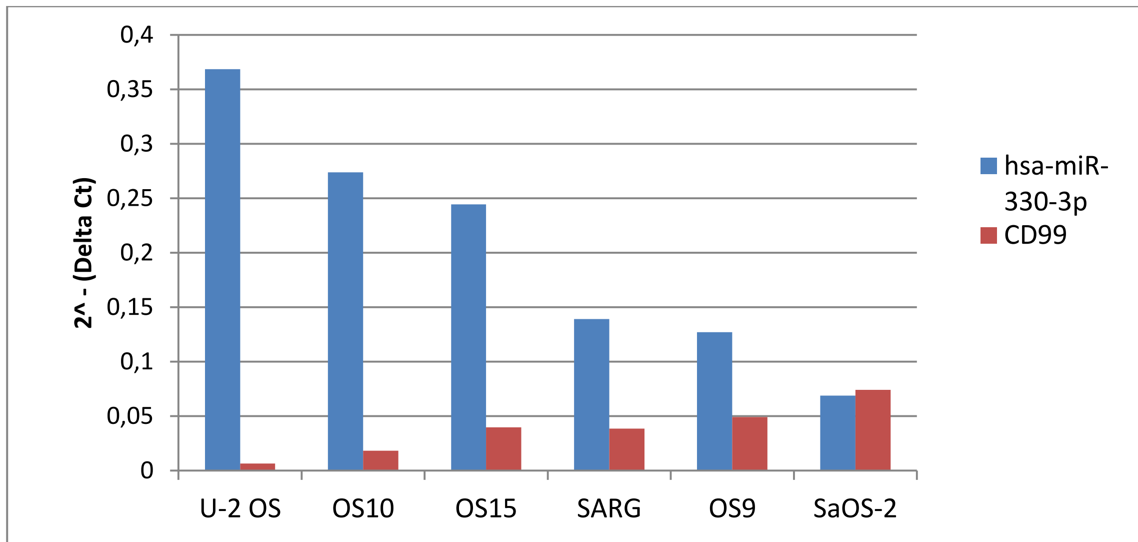

Figure 13. Expression of hsa-miR-330-3p and CD99 in a panel of OS cell lines

Data of miR-330-3p expression in OS samples were available thanks to the RNAseq experiment described in the following chapter. The graphs below show the expression of miR-330-3p and CD99 in OS samples, divided by their status: NED (no evidence of disease) and REL (relapsed). As expected, miR-330-3p expression was higher in patients who show more aggressive tumoral features, whereas CD99 was higher in patients with a better prognosis. It was possible to observe an anticorrelation between the expression of miRNA and target. Unfortunately, this correlation is not significant, probably due to the different techniques used for expression analysis and the small number of samples.

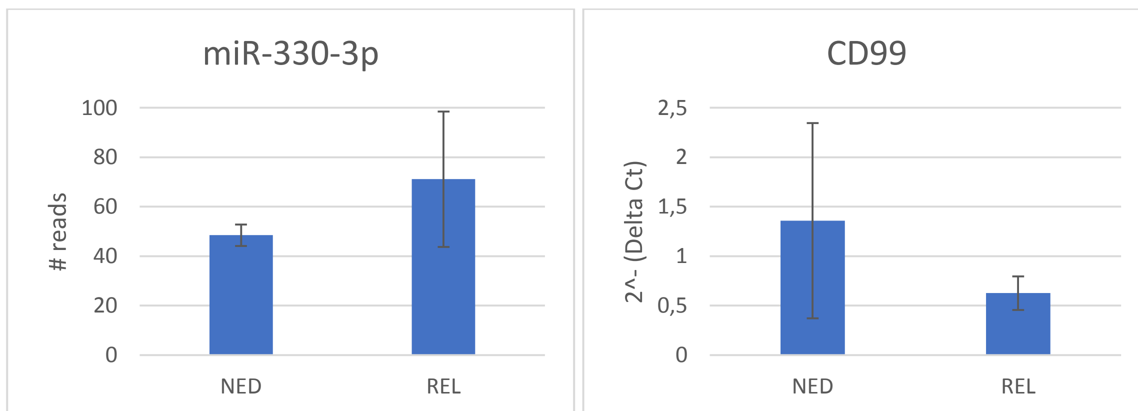

Figure 14. Graphs represent expression of hsa-miR-330-p as number of reads and CD99 as relative abundance in two patients' group (NED and REL)

4.2.3 Functional analysis

Due to the importance of CD99 to regulate malignant phenotype in OS cell line, affecting migration capabilities, the goal of functional studies was to modulate CD99 expression through the treatment with pre-miRNA and antagomiRNA and observe the effects in terms of migration. In U- 2 OS cells, that have high expression of miR-330-3p and are negative for CD99, a treatment for 24 hours with an antagomiRNA increases the mRNA of the target gene (data not shown). Following the transfection, a wound healing assay was performed (Figure 15).

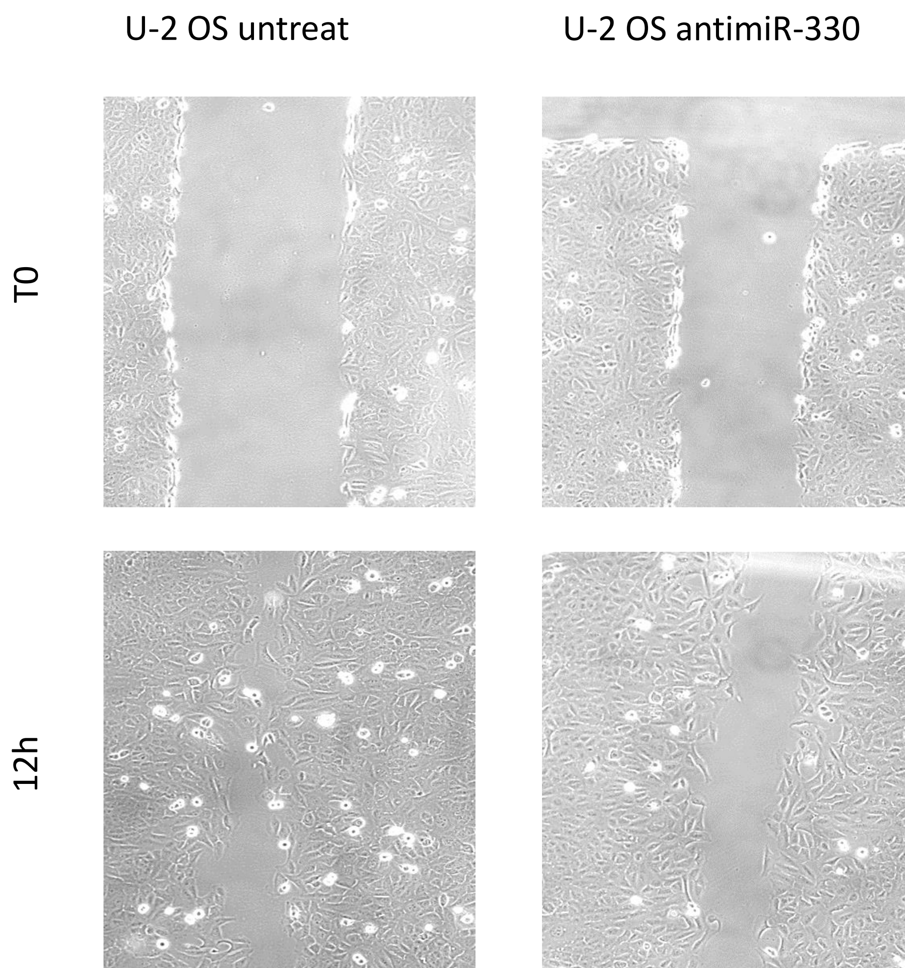

Figure 15. *in vitro* migration scratch after 24h of treatment with antago-miR-330 in U-2 OS cells

As expected, cells treated with the antagomir-330 migrate less than the untreated control, confirming both the targeting of CD99 and its implication in cell migration. The modulation of miR-330-3p is reflected on the signal pathway of CD99, including ROCK2. ROCK2 expression was evaluated by Western Blotting, after 24 hours of transient transfection with antago-miR-330 in U-2OS (Figure 16).

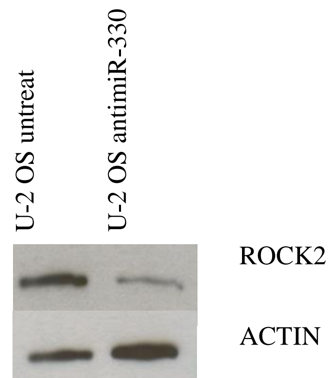

Figure 16. Western Blot analysis of ROCK2 after 24h of treatment with antago-miR-330 in U-2 OS cells

As expected, cells treated with antagomiR-330 showed a decreased expression of ROCK2.

4.3 Identification of a miRNA signature in OS

The third part of the PhD program consisted in the identification of a miRNA signature able to discriminate patients with a better prognosis.

4.3.1 Pilot study: small-RNA sequencing

As a pilot study, a total of 21 samples were submitted for small RNA sequencing, through Illumina Platform. The samples can be divided in 3 biological groups: 7 healthy control like BM-MSCs and human osteoblast, 7 patients with no recurrence or disease progression (NED) and 7 patients relapsed with a local recurrence or a metastasis (REL). Patients were affected by high grade OS, localized at extremities and with no evidence of metastasis at diagnosis. The age at diagnosis was under 40 years and all patients was treated by neoadjuvant protocol. The median of time of recurrence for REL patients is 23 months (range 14-31 months), median of follow up for NED patients is 151 months (range 44-207 months). The bioinformatic analyses were performed to compare NED vs REL.

The following graphs represents the expression of each miRNAs that was found as significantly deregulated in the two examined groups (Figure 15).

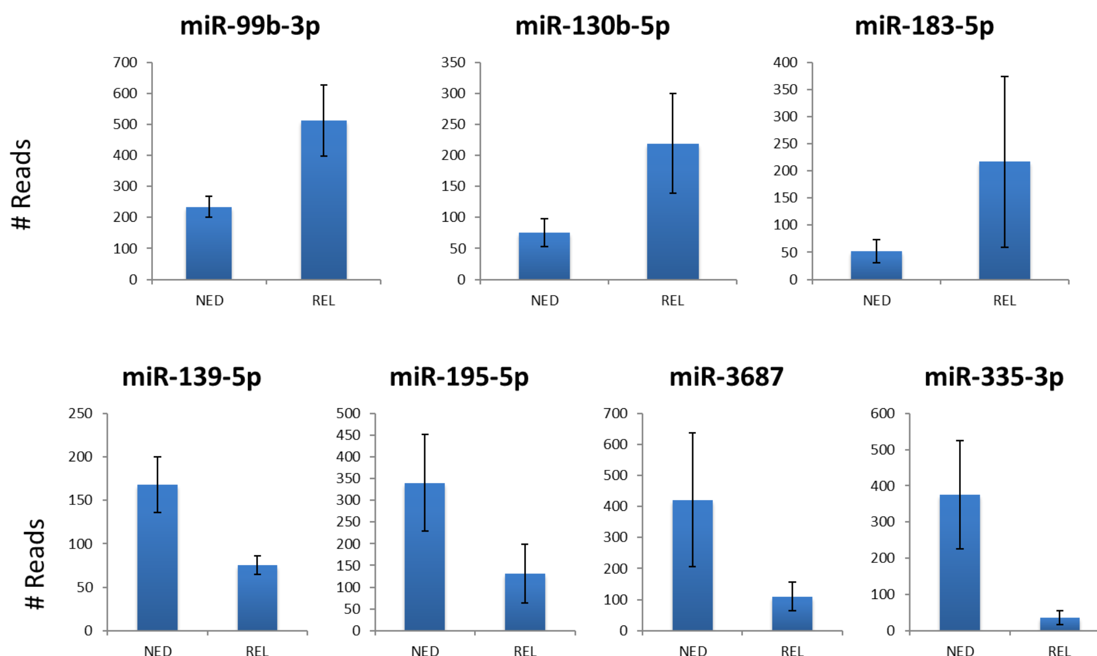

Figure 15. Bar plot graphs represent the mean of reads for each samples groups

In order to verify the quantitative capabilities of the small RNA sequencing, the expression of all the 7 miRNAs was validated through qPCR.

The following table summarizes the fold change data obtained by both the techniques.

miRNA ID	RNAseq linear FC	qPCR linear FC
hsa-miR-183-5p	5.23	6.46
hsa-miR-130b-5p	2.75	1.89
hsa-miR-99b-3p	2.14	1.81
hsa-miR-139-5p	-2.28	-2.28
hsa-miR-195-5p	-3.26	-1.26
hsa-miR-3687	-5.14	1.17
hsa-miR-335-3p	-10.53	-10.40

Table 9. Fold change obtained by analyses of RNAseq data and qPCR data

Except for hsa-195-5p and hsa-miR-3687, the validation through qPCR gives similar results in term of fold change.

4.3.2 Validation study: qPCR

The following validation was conducted on 78 RNA samples derived from OS biopsies: 35 patients belonging to the group of NED, 43 to the group of REL. Patients were affected by high grade OS of the extremities, they did not present evident metastasis at diagnosis and followed a neoadjuvant treatment. The median of follow up of this cohort is 95 months (range 6-310 months).

Quantitative expression analysis was performed using 2^- (delta Ct) method in order to compare the average of two groups.

Three of seven miRNAs were validated and were significantly differentially expressed in the two groups: hsa-miR-99b-3p (Student's t test p-value = 0.001), hsa-miR-130-3p (Student's t test p-value = 0.016), hsa-miR-139-5p (Student's t test p-value = 0.045) (Figure 16).

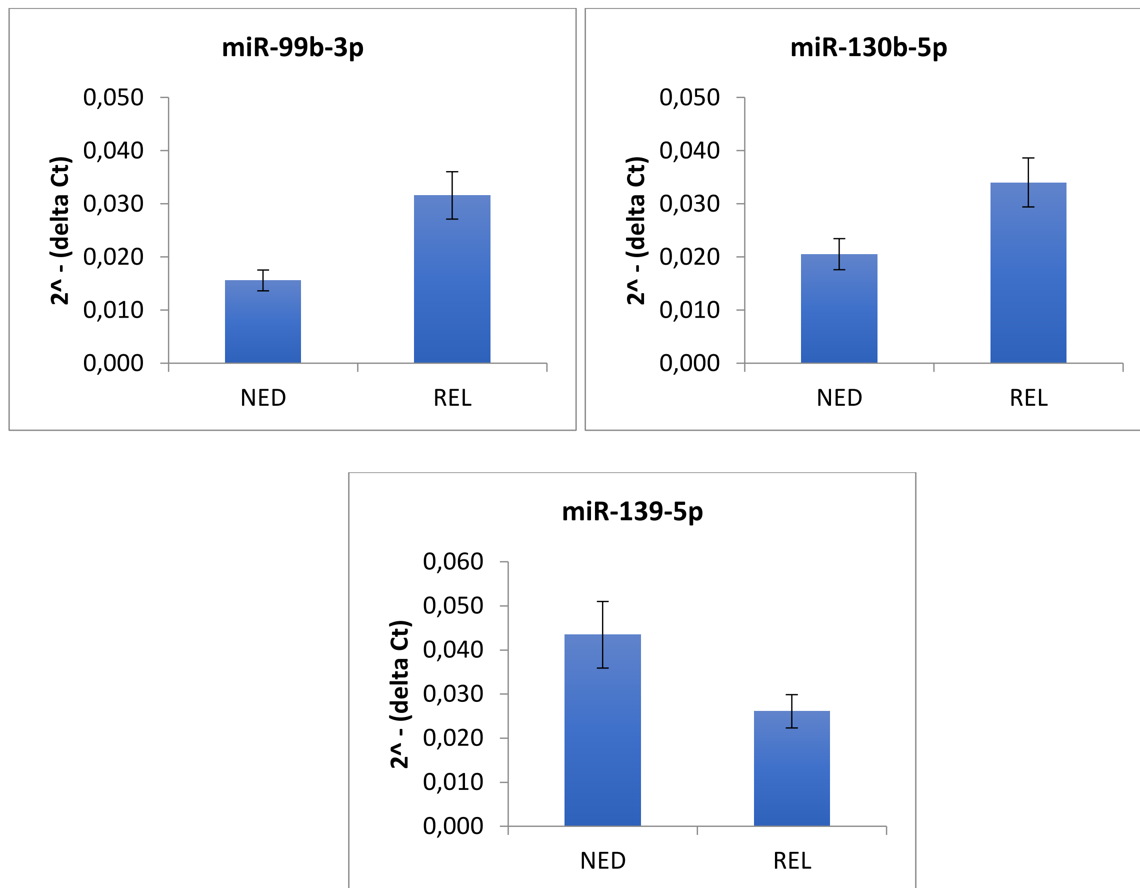

Figure 16. Expression in the two samples group of the 3 miRNAs validated

These three miRNAs are able to discriminate patients with a better prognosis.

4.3.3 Gene expression profiling

To better understand the functions of validated miRNAs, a gene expression profiling experiment was performed using HTA 2.0 Affimetrix microarrays for the same 21 samples used for RNAseq pilot study.

Bioinformatic analyses were then performed. The first analysis regards differentially expressed genes between the two patients' categories, NED and REL. In the meanwhile, miRNA-mRNA prediction analyses were performed for hsa-miR-99b-3p, hsa-miR-130b-5p and hsa-miR139-5p. Only predicted target genes were considered in following analysis. Anticorrelation coefficients were calculated for each miRNA-mRNA couple previously identified. In conclusion, gene set enrichment analyses were run for putative and anticorrelated target genes of miR-130b-5p and miR-139-5p. The following graphs summarize the results obtained by enrichment pathways analysis (Fig 17 and 18).

Each bar represents a pathway resulting enriched, red lines divided not significative pathway from significative one; significance is expressed as -log (p-value).

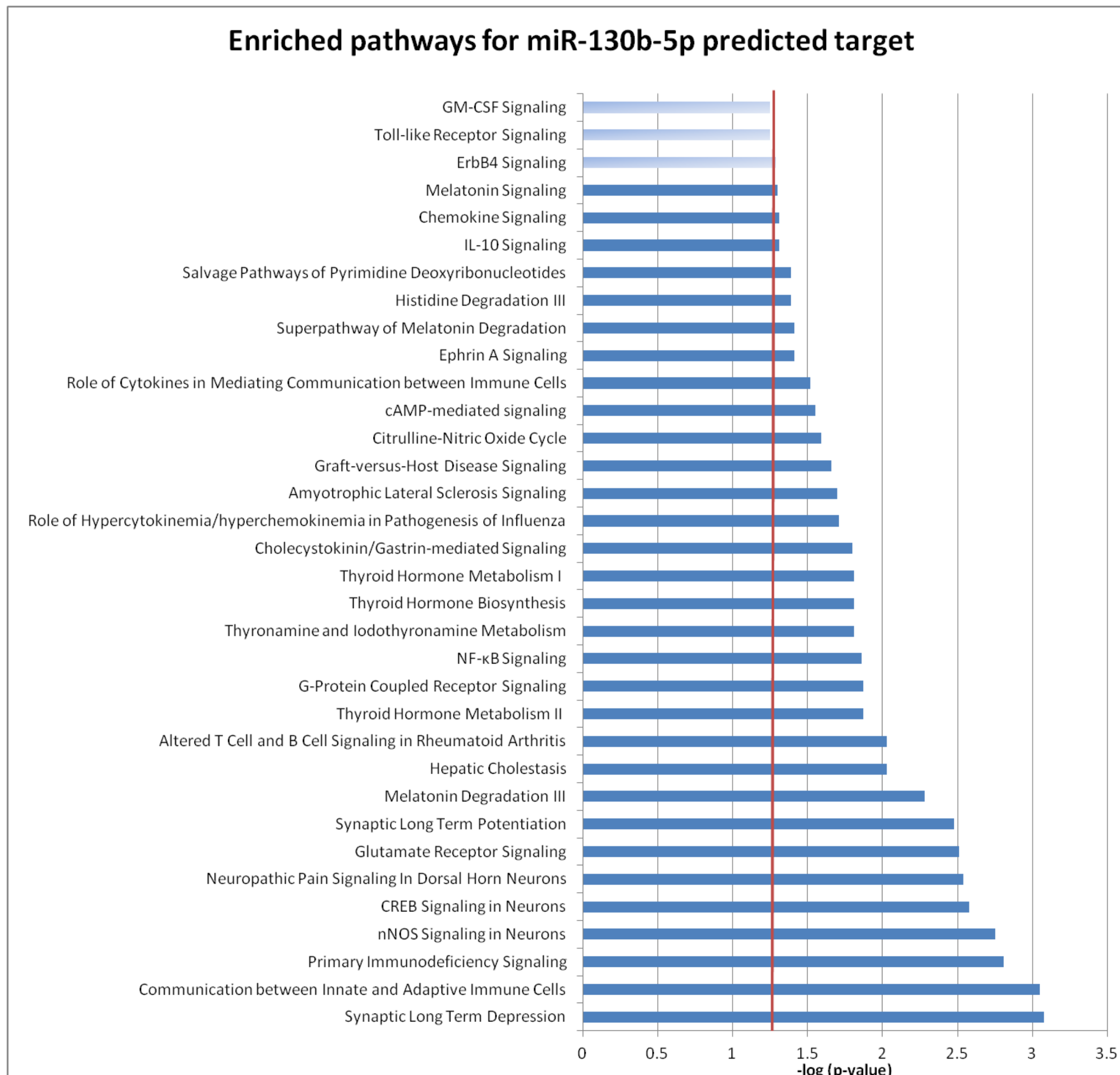

Figure 17. Statistically enriched pathways of predicted target gene of miR-130-5p

Interestingly, different immune response pathways emerged in this analysis.

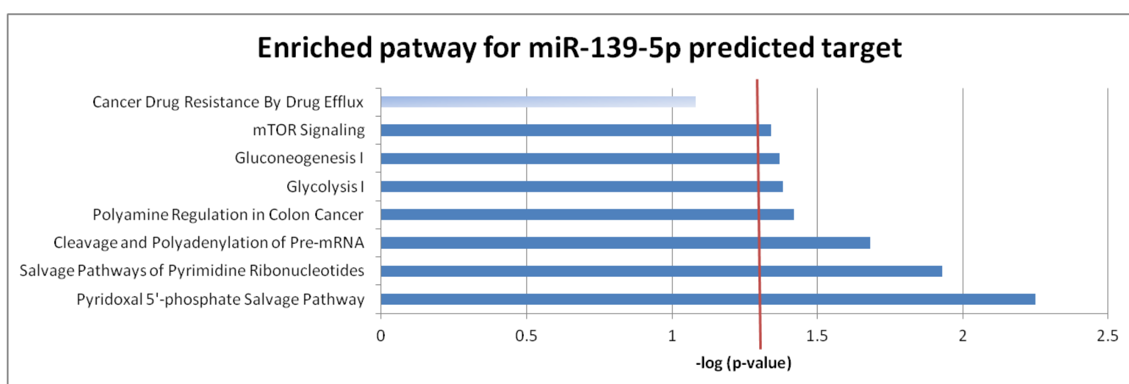

Figure 17. Statistically enriched pathways of predicted target gene of miR-139-5p

These pathways instead are linked to cell metabolism and may be implicated in drugs response.

5. DISCUSSION

Osteosarcoma does not benefit of targeted therapies because it is characterized by high genetic instability and mutation in a broad spectrum of genes. Thus, the identification of a common pathogenetic event is difficult, but it is the key point for the development of targeted therapy. Osteosarcoma is treated with conventional chemotherapeutic agents, which have many short- and long-term side effects. Prognosis of OS patients may be improved through the validation of novel biomarkers, able to predict the response to a therapy regimen. In this manner, patients may be take advantage by more personalized therapeutic strategies, with the aim to limit side effects and improve the survival. A biomarker may be an indicator of a physiological state, of a pathological condition or of the pharmacological response to a therapeutic regimen. The perfect biomarker must have some peculiarities such as high sensitivity, specificity, robustness and predictive value. A non-invasive analysis would be advisable, thus the possibility to evaluate the biomarker in biological fluids, such as urine or blood, is appealing. This study evaluated the role of CD99 as a predictive and prognostic biomarker in osteosarcoma. Prognostic markers could give information about disease outcome independently from the type of treatment; predictive biomarker helps to identify patients who may respond to specific therapeutic regimen (Mc Shane, 2012).

Even if the role of CD99 as oncosuppressor in OS is well documented, only a research group reported that the combination of CD99/HLAII co-expression may be a prognostic indicator in OS (Zhou, 2014). In our cohort, CD99 was associated to prognosis both in the entire cohort and in the arm of patients who received ifosfamide, but lacked its value in the subcohort of patients who did not receive ifosfamide. The involvement of CD99 in ifosfamide sensitivity was confirmed *in vitro* in different cell line models. First, two cell lines established from patients-derived xenograft (PDX) were analyzed for the expression of CD99 and for the sensitivity to 4-hydroperoxy ifosfamide (4-HOO IFO). Differences in IC_{50} doses between the two cell lines are statistically significant; in addition, a higher expression of CD99 corresponds to a greater sensitivity to 4-HOO IFO. This was also confirmed in OS cell lines stably which were transfected to achieve an overexpression of CD99. In fact, in cell lines with a forced expression of CD99, the IC_{50} dose for 4-HOO IFO was significantly lower if compared to parental cell lines. A lower IC_{50} dose matched an increased proportion of apoptotic cells, confirmed by the analysis of the hypodiploid peak after 48 hours of treatment. Unfortunately, few knowledges are available about mechanisms of sensitivity and resistance of ifosfamide in tumoral cells. These processes

may interest: detoxification of ifosfamide metabolites, which involves proteins like aldehyde dehydrogenase (ALDH), glucose-6-phosphate dehydrogenase (G6PD), glutathione (GSH) and glutathione S-transferase (GST); altered uptake and efflux of active metabolites resulting in modulating active transporters such as breast cancer resistance protein (BCRP) and multidrug resistance associated proteins (MRP1, MRP2, and MRP4). Also, DNA repair mechanism may be involved through modification in protein of mismatch repair (MMR) and base-excision repair (BER) complex, or modulation of level of MGMT. Finally, ifosfamide resistance is associated to escape from apoptotic signal resulting in deregulation of both pro-apoptotic (Bcl-2) and anti-apoptotic (Bax) protein (Zhang, 2005). Analyses of some ABC transporters were performed trying to understand the differences revealed in IC_{50}. No significant differences were noticed between cells overexpressing CD99 and parental ones. Therefore, data obtained from in vitro experiments could not totally explain the role of CD99 in increasing sensitivity to ifosfamide in OS. It is plausible an involvement of tumoral microenvironment, in particular of immune infiltrate, in modulating OS sensitivity to this drug.

The involvement of different immunological cell subsets in tumor development and progression and their emerging role in the most effective standard or targeted therapeutic treatments for several tumor histotypes, suggests that understanding composition, function and sensitivity of immune infiltrate may represent a potentially appealing therapeutic option. Unfortunately, the data available for immune infiltrate in OS and its role are still limited. Presence of CD8+ T cells and tumor associated CD68+ macrophages (TAM) is correlated to a better prognosis in the cohort analyzed. Two independent studies confirmed these evidences. A study conducted on 124 OS patients, treated with zoledronic acid, demonstrated the prognostic value of CD8+ T cells (Gomez-Broucher, 2017). As demonstrated by Buddingh, the number of TAM was associated to a better outcome. Also, in a multivariate model, a high number of TAM was associated with the response to chemotherapy, suggesting a role of immune infiltrate in sensitivity to drugs (Buddingh, 2011). The involvement of CD99 in immunological processes leads to study the possible connection between expression of CD99 and markers of immune infiltrates of the tumor. CD99 positive tumors are associated to infiltration by CD8+ and CD68+ immune cells, suggesting a role of CD99 in recruitment of immune cells to tumoral site. Survival analysis demonstrated that the simultaneous presence of CD8 and CD99 (or CD68 and CD99) predicted a better prognosis in the entire cohort; the significance of these analyses was improved when only arm B was considered. These data suggest that CD99 and tumor microenvironment, especially the immune infiltrate, are important for a greater response to ifosfamide. Oxazaphoshorines show immunomodulatory effects, probably due to a bystander effect in the modulation of tumor

microenvironment. This class of chemotherapeutic agents can enhance the antitumor activity of immune cells through several mechanisms, such as homeostatic proliferation, induction of expression of cytokines and removing of suppressor T cells (Proietti, 1997; Binotto, 2003; Bracci, 2007). Homeostatic proliferation leads the maintenance of T cell population by both de novo T cell production in thymus and by the long-term survival of memory T cells (Surh, 2000). As known, CD99 is highly expressed in memory B and T cells (Park, 1999; Yoon, 2003). It is possible to speculate about a role of CD99 in the recruitment of memory T cells at the tumor site, through heterodimerization between CD99 expressed on tumoral cells and on T cells. Additionally, to corroborate the importance of CD99 in response to ifosfamide, in Gougelet *et al.* a panel of 6 miRNAs were identified as essential for the response to this drug and some of these, such as miR-30c and let-7, can target CD99 (Gougelet, 2011).

So, CD99 acts in osteosarcoma as oncosuppressor affecting migration and cell differentiation and its role as a predictive factor has been demonstrated. It is important to have a better knowledge of the regulation of this protein in osteosarcoma. Less is known about transcriptional and post transcriptional regulation of CD99. In fact, only miR-30a-5p is validated for targeting CD99 in Ewing Sarcoma (Franzetti, 2013). In this study, miRNA-mediated downregulation was investigated with the aim to validate new miRNAs able to target CD99 in OS cells. *In silico* predictions led to the identification of several miRNAs, but the choice of miR-23a-5p, miR-27a-5p, miR-190 and miR-330-3p was due to their thermodynamic aspect, type of seed sequence, and presence of multiple sites in the 3'UTR of CD99. The targeting was confirmed for miR-23a-5p, miR-27a-5p and miR-330-3p through luciferase assays in recipient cells and by qPCR and Western Blotting in the model of interest. Functional studies were conducted only for miR-330-3p because of its capacity of giving a stronger silencing of CD99 in OS cells. Moreover, basal expression of this miRNA anticorrelated with CD99 expression in a panel of OS cell lines. Functional studies showed that miR-330 affected migration and modulated expression of ROCK2. miR-330-3p has therefore an oncogenic role in osteosarcoma. Evidences about the role of miR-330-3p are contradictory. Like other miRNAs, it may act both as oncomiR and oncosuppressor miRNA, depending on cell types. In Chronic Fatigue Syndrome, upregulation of miR-330 induces a decrease of MMP9 protein level, confirming the role of this microRNA in cellular migration (Petty, 2016). Also in Non-Small Cell Lung Cancer (NSCLC), miR-330 has oncogenic role, confirmed by a higher expression in tumoral samples comparing to normal samples. miR-330-3p induces the downregulation of early growth response 2 (EGR2), leading in cell proliferation *in vitro* and cell cycle progression in NSCLC (Liu, 2015). Moreover, in NSCLC miR-330 expression is associated to tumor invasion and brain metastasis growth through

the activation of MAPK/ERK pathway and directly targeting GRIA3 (Wei, 2017). The same effects observed in NSCLC about the downregulation of EGR2 were confirmed in osteosarcoma model by Zhuo and colleagues (Zhou, 2015). In glioblastoma, miR-330-3p regulate cell migration and invasion ability through downregulation of SH3GL leading in activation of PI3K/Akt and ERK modulation. In breast cancer, miR-330-3p promotes metastases development through targeting of CCBE1 (Mesci, 2017). Also in hepatocellular carcinoma (HCC), a high expression of miR-330 correlates with poor prognosis. miR-330 promotes cell growth and invasion targeting ING4, a gene involved in modulation of gene transcription via interaction with histone acetyltransferase and histone deacetyltransferase (Hu, 2016). Finally, in esophageal cancer miR-330 interacts with Programmed Cell Death 4 (PDCD4). The main effects of this interaction are the increase of cell growth, cell migration, invasion and inhibition of cisplatin-induced apoptosis (Meng, 2015). In our data set deriving from a pilot study on small RNA sequencing, miR-330 is expressed at higher levels in relapsed patients in comparison to those who remained continuously disease-free. Unfortunately this difference is not significant, probably due to the small cohort analyzed. Taken together, these evidences may corroborate a tumor-inducing role of miR-330 in OS.

The pilot study on Small RNA sequencing consisted in analyzing 3 biological groups composed by 7 RNA samples from healthy controls (bone-marrow mesenchymal stem cells and osteoblasts), 7 extracted from biopsies of NED (no evidence of disease) patients and 7 from REL (relapsed) patients. The goal was to identify miRNAs and others small RNAs able to discriminate these subgroups, in particular NED versus REL. Several studies on miRNA signature are available in literature. The first OS signature was identified by Jones, that analyzed a total of 18 OS samples using microarray-based techniques. They identified 34 miRNAs deregulated in OS specimens in comparison to normal tissue, providing a miRNome associated to OS pathogenesis (Jones, 2012). A second miRNA signature was discovered in a cohort of 27 patients of Mexican-American ethnicity, affected by high grade OS of extremities. Expression analyses of 754 miRNAs were performed through High-throughput RT-qPCR. They found 42 miRNAs that were associated to survival in OS (Sanchez-Diaz, 2014). The last miRNA signature discovered consists in 29 miRNAs. The discovery set of 23 patients were analyzed for the expression of 752 miRNA through High-throughput RT-qPCR. These miRNAs were analyzed on 101 samples. This is the largest cohort published for this type of studies (Andersen, 2017). In our data set, for the first time small RNA sequencing was performed with the aim to discover a miRNA signature. It is also possible to identify other differentially expressed small RNAs in our cohort, such as piRNA, snoRNA, tsRNA. Bioinformatic analyses revealed a list of 7

significantly deregulated miRNAs in the two patients' groups. The validation was then conducted by qPCR in a larger cohort composed by 35 NED and 43 REL patients. Three of seven miRNAs were validated and were significantly differentially expressed between the two groups: hsa-miR-99b-3p (Student's t test p-value = 0.001), hsa-miR-130-3p (Student's t test p-value = 0.016), and hsa-miR-139-5p (Student's t test p-value = 0.045). miR-99b-3p and miR-130b-3p were upregulated in REL, suggesting an oncogenic role; conversely, miR-139-5p was downregulated in REL, in line with a possible oncosuppressor function. The better-understood miRNA is hsa-miR139-5p, while less or nothing is known for the other two.

A revision of literature showed that miR-139-5p has oncosuppressive features in several tumor types, like hepatocellular carcinoma, breast cancer, esophageal cancer, bladder cancer, colon rectal cancer, acute myeloid leukemia (Wong, 2011; Liu, 2013; Song, 2014; Xu, 2016; Luo, 2017; Krowiorz, 2016). miR-139-5p exerts its tumor suppressive role at different levels, such as cell growth and apoptosis, cellular migration, and invasion. In NSCLC miR-139-5p suppresses cell growth, metastasis formation and induction of apoptosis through direct targeting of IGF1R, CCDN1, KIP27, MMP7, MMP9 and c-MET (Xu 2015; Sun, 2015). Two independent studies demonstrated that miR-139-5p is less expressed in colonrectal cancer in comparison to the normal counterpart, moreover low expression of this miRNA is associated to poor prognosis. miR-139-5p inhibits cell migration and invasion through targeting Notch1 (Song, 2014). miR-139-5p is also involved in sensitivity to oxaliplatin and fluorouracil in multidrug resistant cell lines of CRC. Low expression of the miRNA makes cells more resistant to chemotherapeutic agents and this effect may be partially reverted through overexpression of Notch1. These data confirm the importance of miR-139-5p Notch1 axis in tumorigenesis and chemoresistance in CRC (Xu, 2016). miR-139-5p can modulate invasion and migration abilities *in vitro* thanks to direct targeting of ROCK2. miR-139-5p ROCK2 targeting has been confirmed in three different tumoral model, such as hepatocellular carcinoma, ovarian cancer and colorectal cancer (Wong, 2011; Wang, 2017; Shen, 2014). Few studies are available for miR-99b-3p and miR-130b-5p. The role of miR-99b-3p was studied in oral squamous cell carcinoma (OSCC) in which it has oncosuppressive characteristics thanks to the inhibition of glycogen synthase kinase-3β (GSK3β). Indeed, downregulation of GSK3β leads to the inhibition of OSCC cell proliferation and suppression of p65 (RelA) and G1 regulators (cyclin D1, CDK4 and CDK6) *in vitro* (He, 2015). miR-130b-5p belongs to a 25 miRNAs signature of triple negative breast cancer; miR-130b-5p is more expressed in tumor samples in comparison to normal adjacent tissue and exerts its oncogenic role through direct targeting CCNG2. Inhibition of CCNG2 induces cell proliferation (Chang, 2015). In human epithelial ovarian cancer (EOC), miR-130b-5p is

overexpressed in tumoral tissues in respect to normal ones (Wang, 2014). The data available about miR-99b-3p and miR-130b-5p contrast with the possible role of these molecules in OS.

For understanding the biological role of these miRNAs in osteosarcoma a gene expression profiling experiment was conducted on the same 21 samples. The analyses were conducted in the two patients' groups: differentially expressed genes between NED and REL were compared to the lists of potential targets for each miRNA validated. Finally, enrichment pathway analyses were performed for a greater comprehension of the biological function of these miRNAs in OS models. miR-130b-5p seems to be involved in the interaction with immunological processes. In fact, 12 of 31 significantly enriched pathways involved immune cell communication or pathways proper of immune system, like communication between innate and adaptive immune cells, NF-kB signaling, and IL-10 pathway. In particular, IL-33 seems to be an important target of miR-130b and it is also found as part of the 9 pathways emerged by enrichment analyses. IL-33 belongs to the IL-1 family and its cognate receptor is ST2 (suppression of tumorigenicity 2). IL-33 has a key role in tissue homeostasis and responses to environmental stresses; it acts as an alarmin secreted by damaged and necrotic cells. It is constitutively expressed in almost all cell and tissue types (Liew, 2016). Loss of IL-33 is associated to metastases development in carcinomas through a mechanism of immune surveillance escape (Saranchova, 2016). Moreover, low expression of IL-33 is associated to a poor prognosis in OS (Koster, under review). miR-139-5p seems to be involved in cellular metabolism, as demonstrated by enriched pathways such as glycolysis, gluconeogenesis and mTOR signaling. Moreover, pathways like salvage pathway of pyrimidine ribonucleotides and pyridoxal 5'-phosphate are enriched, suggesting a role of miR-139-5p in resistance to classical chemotherapeutic agent.

In conclusion, the predictive and prognostic role of CD99 in OS has been reported. In clinical samples, CD99 acts as a predictive biomarker for ifosfamide response. CD99 is associated with an increased sensitivity in OS cell lines, corresponding to a greater apoptosis rate. CD99 tumoral expression correlates to an enrichment in CD8+ and CD68+ cells in OS samples. Taken together these data confirm a role of CD99 in ifosfamide sensitivity in osteosarcoma, thus CD99 is able to increase both the cytotoxic and the immunomodulatory effects of ifosfamide, making osteosarcoma cells more sensitive. The study regarding post-transcriptional regulation of CD99 identifies hsa-miR-330-3p as a modulator of CD99 expression. Moreover, miR-330-3p affects downstream effector of CD99, like ROCK2, leading to a modulation of migration capabilities. Finally, a 3-miRNA signature (hsa-miR-99b-3p, hsa-miR-130b-5p and hsa-miR-139-5p), identified by small RNA sequencing, discriminates patients who remained disease-free from those who relapsed. Enrichment pathway analyses showed the involvement of these miRNAs in

immunological processes and cellular metabolism, underlining the importance of these mechanisms in the pathogenesis and response to therapy in OS.

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