ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

ONCOLOGIA, EMATOLOGIA E PATOLOGIA

Ciclo 36

Settore Concorsuale: 06/D3 - MALATTIE DEL SANGUE, ONCOLOGIA E REUMATOLOGIA

Settore Scientifico Disciplinare: MED/06 - ONCOLOGIA MEDICA

DECIPHERING THE ROLE OF IL-1 SIGNALING AS AN ESCAPE PATHWAY IN EGFR-DEPENDENT HEAD & NECK SQUAMOUS CELL CARCINOMA (HNSCC): IMPLICATIONS FOR TREATMENT

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Esame finale anno 2024

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1. ABSTRACT

Tumours stemming from the oral cavity, pharynx, larynx, salivary gland, and nasal cavity are collectively placed under Head and Neck Cancer (HNC). Overexpressed EGFR accounts for nearly 90% of Head and Neck cancer. Cetuximab – a chimeric monoclonal antibody IgG1 (FDA approved – 2006) was designed explicitly to target the extracellular domain III of the EGFR to curtail tumor proliferation. Cetuximab + radiation is the standard therapy for metastatic Head and Neck Cancer. Cetuximab induces ADCC-mediated cell death, yet, prolonged exposure leads to acquired resistance toward the drug ultimately leading to relapse. Relapse is marked by the aggressive progression of the disease and with a poor survival rate. Patient data acknowledge the role of IL-1 signaling in determining the overall outcome in regard to disease progression and overall survival in patients. Such a tight nexus between IL-1 signaling and the EGFR signaling pathway makes the current treatment strategies fall short of achieving the desired outcome. Thus, it becomes imperative to establish the crucial role played by IL-1 cytokines in determining the overall tumorigenesis.

In our study, the main intent is to establish the sort of role played by IL-1 signaling and to overhaul the current treatment strategies with the intention of improving their efficacy. To commence with, we employed the FaDu tumor cell line (marked with high expressing EGFR) and combinatorial drug action by employing both anti-EGFR and anti-IL-1/IL1R1 inhibitors concurrently and measuring the treatment efficacy. Additive drug action was found to be more effective in the arrest of overall tumor proliferation compared to mono-therapy (anti-EGFR) alone. From a holistic view, we ascertain that combinatorial drug action was found to be effective compared to monotherapy using classical anti-EGFR. Independently, we aimed to define the efficacy of our laboratory-designed anti-IL-1 decoy receptor named human TRAP IL-1 in neutralizing the free IL-1 ligands and the aftermath effects in cells. The results demonstrated the neutralization of IL-1 by TRAP IL-1 besides EGFR boiled down the overall proliferative potential of tumour cells.

Summarizing our finding, we verified that IL-1 aids and abets in the proliferation of tumor cells alongside EGFR signaling, thus making both EGFR and IL-1/IL1R1 coupled targets. Hence, the simultaneous blocking of both EGFR and IL-1/IL1R1 signaling indeed increased the overall efficacy of the treatment in vitro cell line model, yet the efficacy in vivo model is yet to be determined. Convincingly, amending the current treatment strategy with the induction of anti-IL-1/IL1R1 on par with anti-EGFR might be fruitful in increasing Progression-Free Survival and Overall Survival in patients.

2. INTRODUCTION

BACKGROUND

Cancer is regarded as one of the deadliest diseases and leads the table as the most common cause of death globally. From the notion of "bad luck" to the present-day scenario, major advancements in scientific techniques have made significant strides in understanding the mechanisms of the precise origin of cancer (1). Technological advancements in the field of molecular biology have substantially improved the efficacy of cancer diagnosis and treatment strategies, despite the drastic shifts in individual lifestyles and surrounding environmental factors that have contributed to an increase in cancer diagnosis and related deaths around the world (2). *Hannah et al.*, classified the multimodal characteristics of Cancer into six major "Hallmarks" based on their degree of complexity, and further classified the additional characteristics as enabling and emerging factors (3, 4).

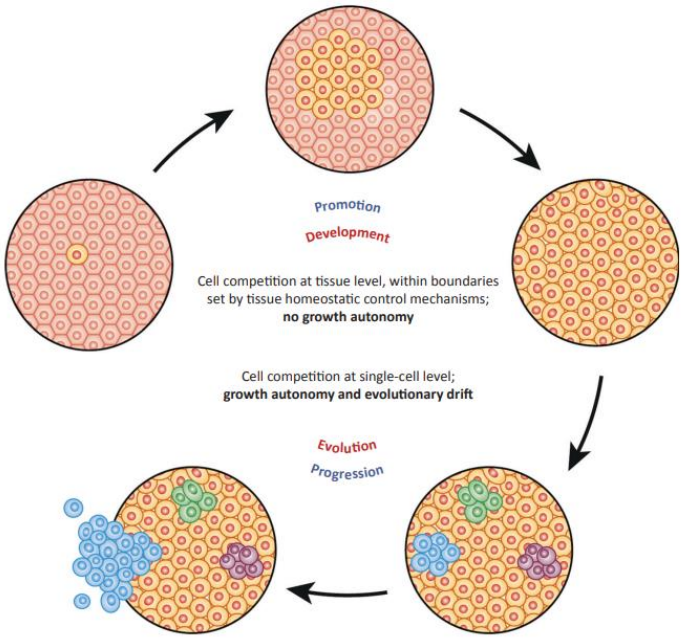


Fig: Multistep origin of tumour from normal cells to advanced invasive and metastatic stage (an overview). Adapted from (5)

Genetic mutations and environmental factors are primarily responsible for the malignant transformation of normal tissues into cancer (6) and the process of such malignant transformation involves several steps, starting from the benign stage to a full-blown malignant transformation (7). An increased prognosis associated with early cancer detection and treatment has been linked to an increase in patient survival rate (8).

2.1 HEAD AND NECK CANCER

Head and Neck Squamous Cell Carcinoma (HNSCC), also known as Head and Neck Cancer (HNC), is the ninth most common cancer worldwide and is a diverse group of tumours that originate from the aero-digestive tract. Squamous cells of epithelial origin are the main origin of tumours comprising 90% total of Head and Neck Cancers (9). Treating HNC can be difficult due to its relative heterogeneity in nature, delayed diagnosis, and intermittent nature of disease, particularly when the tumour is diagnosed at a later stage in life (10). Similar to other tumours, HNC is initiated from a genetic disorder that develops gradually and is characterized by a build-up of subsequent genetic mutations that progress from precancerous lesions to highly invasive carcinoma. Though there is increased scientific progress in treating the disease, a median 5-year survival rate for such an aggressive tumour has been reported (11).

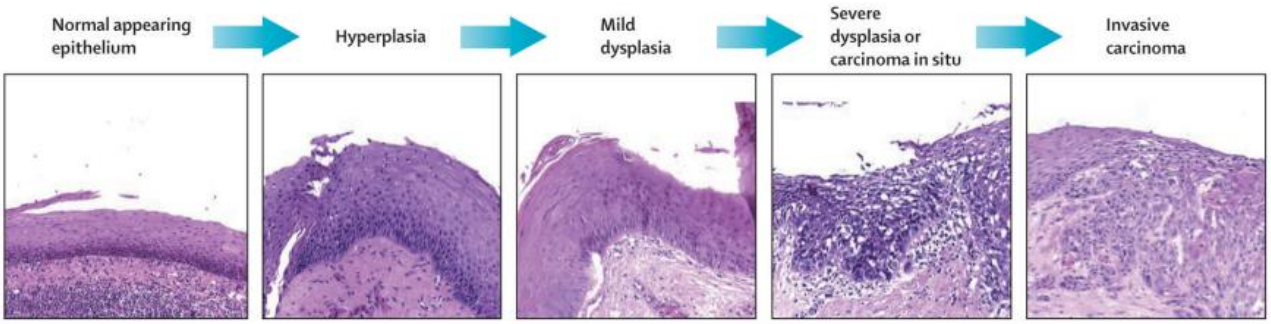


Fig: Step-wise progression of Head and Neck Squamous Cell Carcinoma from normal epithelial cells. Adapted from (12).

2.1.1 EPIDEMIOLOGY:

A large proportion of Head and Neck Cancer cases are often diagnosed at a progressive stage, with an average of almost 6,00,000 cases reported across the globe. According to epidemiological research, young white individuals are at a higher risk of developing Oral Squamous Cell Carcinoma (OSCC) (13). It is commonly known that using tobacco products and consuming alcoholic beverages over an extended period might trigger the development of Head and Neck Cancer. Furthermore, Genome-wide Associated Studies (GWAS) indicate a positive correlation between genetic variations/mutations in alcohol-metabolizing genes linked to the progress of Head and Neck Cancer (14). In addition to known etiological factors, viral infection with the Human Papilloma-Virus (HPV) further escalates the risk of the development of Head and Neck Cancer. Of the numerous known HPV variants, variant 16 is strongly correlated to the majority of known HNC cases, whilst, variant 18 is present in almost 90% of HPV-related Oropharyngeal Carcinoma cases (15).

2.1.2 STAGING AND DIAGNOSIS

Deciphering the tumour stage greatly depends on the anatomy and tumour burden. According to the American Joint Committee on Cancer (AJCC) and the Union of International Cancer Control (UICC), the staging of tumours is principally based on the TNM classification. Such TNM classification is derived by assessing the primary site of the tumour origin, lymph node involvement, and extent of metastatic spread of the tumour (16). Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) act as primary tools in establishing the tumour stage. Accuracy in tumour staging can be further enhanced by deploying advanced tools like FDG-PET CT scan that proved effective in detecting recurrent disease manifestation and predicting the extent of distant metastatic spread of the disease (17). There is often a negative correlation between a patient's overall survival rate and a delayed diagnosis of the disease. Early tumour detection can be delayed for several reasons. While some factors, such as ignorance and fear of the disease, are considered irrational, they are still thought to have an impact on the delayed diagnosis (18). Detection of prognostic biomarkers, such as non-coding RNAs like tRNA halves, YRNA fragments, long non-coding RNA (lnc RNA), and micro RNA (miRNA), from the patient's blood and saliva, in addition to conventional diagnostic parameters, helps to determine the detection and potential diagnosis of the tumour and provides additional observational information regarding the severity of the disease (19).

2.1.3 TREATMENT

The two main therapeutic modalities used to treat locally advanced Head and Neck Cancer are radiation therapy and surgical tumour resection. Radiation, surgery, and chemotherapy are used in combination to treat patients with metastatic spread (20). A recent development in radiation therapy is the addition of proton therapy (72). Due to the over-expression of EGFR, the FDA-approved cetuximab, a chimeric monoclonal antibody, in 2006 used to treat metastatic colorectal cancer, Head and Neck Squamous Cell Carcinoma. Tyrosine Kinase inhibitors, such as gefitinib, erlotinib and afatinib are also used to inhibit the EGFR mediated down-stream signaling in Head and Neck Squamous Cell Carcinoma (21,22).

2.2 ERBB FAMILY

Normal cell signaling is crucial for maintaining cellular homeostasis, and any disruption associated with it may negatively influence and disrupt a variety of cellular processes, including growth, proliferation, and metabolism posing a serious risk to cellular homeostasis and may initiate the development of a tumour (23). The tyrosine kinase-mediated signaling of the ERBB receptor family, was initially discovered in the avian erythroblastosis tumour virus. The importance of ERBB-mediated signaling in cancer research has become more widely recognized since the 1980s. Four ERBB family receptors with varying functions—from cell-cell interaction in organ development to a variety of other functions have been identified as a result of subsequent research. From the evolution point of view, ERBB receptor-mediated signaling family members have shown a high degree of conservation in their signaling hierarchy (24).

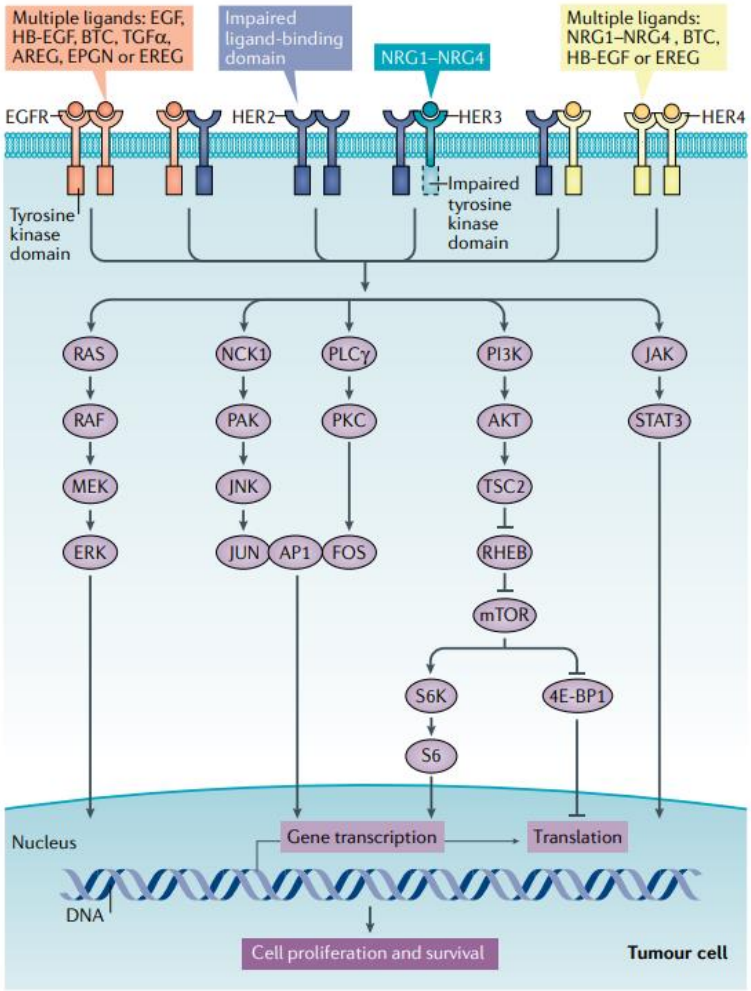


Fig: The RAS/MAPK, PI3K/AKT, and PLCy pathways are the three-primary cell signaling pathways for the ERBB family of receptors. Adapted from (26).

ERBB activation is triggered by both heterodimerization and homodimerization of receptors. However, genetic modifications in the ERBB family may contribute to carcinogenesis, particularly in solid tumours. The aberrant activation of EGFR and HER2 receptors in different solid tumours is boosted by genetic altercations such as gene amplifications, point mutations, and deletions (25,26). Receptor-specific ligands trigger the activation of receptor-mediated signaling. Receptor-specific ligands are ligands that bind only to a single receptor; examples of these include classical ligands like TGF-α and EGF that bind only to ERBB1/EGFR. Ligands with dual binding specificity, such as Neuregulin 1 and 2, have the ability to bind to both ERBB 3 and ERBB 4 and initiate a downstream signaling cascades. Nevertheless, there is no known ligand that is specific to the HER2 receptor. One of two processes: homodimerization or heterodimerization—activates this receptor and triggers the downstream receptor-mediated signaling cascade (27).

	ErbB-1	ErbB-2	ErbB-3	ErbB-4
EGF	+	-	-	-
TGF-a	+	-	-	-
Amphiregulin	+	-	-	-
HB-EGF	+	-	-	+
Betacellulin	+	-	-	+
Epigen	+	-	-	+
Epiregulin	+	-	-	+
Neuregulin-1	-	-	+	+
Neuregulin-2	-	-	+	+
Neuregulin-3	-	-	-	+
Neuregulin-4	-	-	-	+
Neuroglycan C	-	-	+	-
Tomoregulin	-	-	-	+

Table: Receptor-specific ligands and their corresponding receptors in the ERBB receptor family Adapted from (27)

2.2.1 MAJOR SIGNALING PATHWAYS BY ERBB FAMILY

The ERBB receptor family is a vital player in initiating multiple cell signaling pathways that are crucial for a variety of cell functions.

- Mitogen-activated RAS-mediated pathway plays a role in a variety of pathways ranging from cellular proliferation, migration, differentiation, and apoptosis (28).

- The Akt/mTOR pathway mediated by PI3Kinase is essential for initiating the signaling pathways that govern cell cycle progression and cytoskeletal reorganization in cells (29).

- The JAK/STAT pathway has a significant impact on the cytokine-mediated cell signaling response and is further crucial for the development of tumours (30).

2.2.2 ROLE OF EGFR-MEDIATED SIGNALING IN HEAD AND NECK CANCER

Several studies have demonstrated an association between HNSCC and EGFR overexpression. Additionally, such research studies have further demonstrated that treatment strategies play fairly better when conventional radiation therapy is often combined with EGFR-inhibiting monoclonal antibodies, which has been shown to outperform radiation therapy alone, thus providing additional proof of the close relationship between the onset of Head and Neck Cancer and over-expressed EGFR (31).

In addition to somatic point mutations, Head and Neck Cancer is known to frequently harbour EGFRvIII mutations, a particular type of EGFR mutation that leads to deletion of exons 2-7 and renders the mutant receptor incapable of binding any known ligand. Increased cellular proliferation is observed in tumour cells harbouring this particular mutant type along with greater invasive and metastatic potential (32). Research studies have demonstrated that the over-expression of EGFR can be explained by the genetic amplification of EGFR (33). The signaling network triggered by EGFR further becomes complicated by the involvement of new/additional players contributing towards tumourigenesis. One such player is the activation of the c-Met receptor activated by the Hepatocyte Growth Factor Ligand (HGF) in the development of Head and Neck Cancer. c-Met receptor is known to trigger Akt/mTOR and RAS pathways, thus providing one additional role in the proliferation of Head and Neck Cancer. Genetic array data further ascertain the role of overexpressed c-Met towards the recurrence of locoregional Head and Neck Cancer (34).

The PI3K pathway, which is triggered by EGFR signaling, is another complex pathway that is thought to be critical to the development of Head and Neck Cancer. It has been observed that PI3K mutations are mutually exclusive with EGFR amplification, as they do not correlate with each other. PIK3Ca mutations are expected to have a substantial role in the carcinogenesis of HNSCC and have the potential to be oncogenic (35). The invasive potential of tumours is found to be closely correlated with both genetic amplification and overexpression of PIK3Ca. However, genetic amplification of the PIK3Ca gene alone has a limited capacity to initiate such a complex mechanism towards carcinogenesis in Head and Neck Cancer (36). The PIK3Ca pathway serves to be a potential target in cancer treatment owing to its greater significance in facilitating tumour progression;

however, the development of mutation specific inhibitors become inevitable considering the signaling nexus of PI3K-Akt pathway in both normal cells and tumour cells (37).

The discovery that the RAS gene as a potent oncogene in the aetiology of Head and Neck Cancer catalysed additional investigations. Several investigations have demonstrated that H-RAS is the gene most commonly found to be mutated, compared to K-RAS and N-RAS, with a higher degree of mutations found in the G12, G13, and Q61 regions respectively (38).

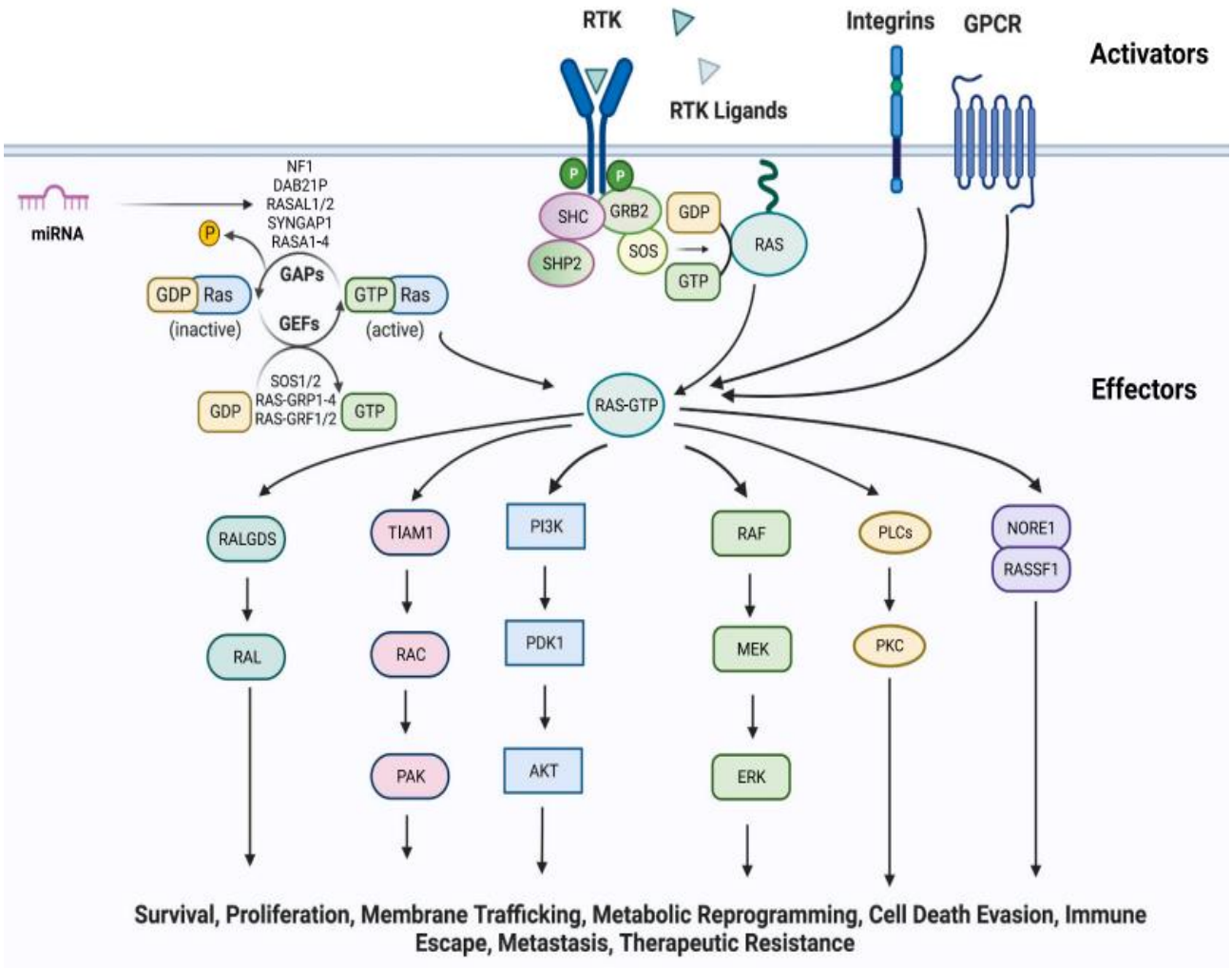


Fig: RAS-mediated signaling cascades driving the activation of various cellular pathways. Adapted from (38).

The relationship between HPV status and mutated H-RAS is a major factor in assessing the degree of validity in Head and Neck Cancer. Such associations have also been observed in cervical cancer, indicating a synergistic relationship between H-RAS mutation and HPV status in

carcinogenesis (39). Based on current data analyses, it is highly likely that the signaling network possessed by RAS-mediated pathways in HNC is highly destructive in nature; therefore, treatment strategies that target the RAS-driven progression of HNC must be enhanced (38).

2.3 TUMOUR MICROENVIRONMENT AND CANCER

For several decades, there has been a philosophy that tumours develop due to the inherent features of the tumour cells. As a consequence, the majority of studies have concentrated towards this theory, giving little to no consideration to the surrounding factors that assist in the development of the tumour. Due to a more comprehensive understanding of the genesis of tumours, research on the immediate environment surrounding tumour cells has gained momentum since the late 1970s. Tumour-microenvironment refers to the immediate surroundings of the tumour cells and provides insights into additional non-genetic factors that play an integral role in tumourigenesis (40). There are many different actors in the tumour microenvironment, and it is now crucial to talk about how each of these actors specifically affects the growth of tumours. It is widely known that several factors, including extracellular matrix, natural killer cells, cancer-associated fibroblasts, cytokines, tumour-associated macrophages, and lymphocytes infiltrating the tumour, are greatly associated and proved essential for the development of tumours and collectively bundled under Tumour-Microenvironment (41). Chronic inflammation plays a crucial role in carcinogenesis among the many known contributing parameters and the inflammatory cells that make up the tumour-microenvironment are primarily responsible for the initiation and development of tumours. This biological relationship has enabled naïve tumour cells to perform a wide range of functions, such as tumour migration, proliferation, and other neoplastic transformations. Cytokines with pro-inflammatory potential such as IL-1α and TNF-α, which are secreted by activated macrophages, play a crucial role in the development of melanoma by inducing VEGF-a and IL-8 in melanocytes. Such a process of vascular angiogenesis is essential for providing a sufficient supply of nutrients for the stable and continuous growth of neoplastic cells (42).

2.3.1 ROLE OF IL-1 SIGNALING IN CANCER

As we previously indicated the crucial contribution of chronic inflammation towards the growth of tumours, new research studies have demonstrated the distinctive function of interleukins as an essential and integral component leading to bolstering therapeutic drug resistance. It is extremely difficult to precisely identify the action of the different players within the Tumour-Microenvironment due to the meticulous coordination of IL-1-driven tumourigenesis amongst cells present in the TME (43). A close relationship exists between members of the Toll-Like Receptor (TLR) family ligands and receptors that make up the IL-1 cytokine family (44). Synthesis of IL-1 by tumour or host cells is considered to be a potential cytokine with an intriguing capacity to play a detrimental role in tumourigenesis.

IL-1 family	Specific receptor	Coreceptor	Function
IL-1α, IL-1β	IL-1R1	IL-1R3	Pro-inflammatory
IL-1β	IL-1R2	IL-1R3	Anti-inflammatory
IL-1Ra	IL-1R1	NA	Anti-inflammatory
IL-18	IL-1R5	IL-1R7	Pro-inflammatory
IL-33	IL-1R4	IL-1R3	Pro-inflammatory
IL-36α, β, γ	IL-1R6	IL-1R3	Pro-inflammatory
IL-36Ra	IL-1R6	IL-1R3	Anti-inflammatory
IL-37	IL-1R5	IL-1R8	Anti-inflammatory
IL-38	IL-1R6	IL-1R9	Anti-inflammatory

Table: IL-1 family and their corresponding immune function. Adapted from (44).

IL-1 is channelled either by direct or indirect means in inducing angiogenesis and metastasis thus increasing the invading potential of the tumour cells (45). The significance of the IL-1/IL1R1 signalling axis in regards to HPV status in Oro-Pharyngeal Carcinoma (OPC) cells has been demonstrated by *Sarmad Al-Sahaf et al.*, characterizing that OPC cells with positive HPV status had negligible to no chemokine expression, while, OPC cells with negative HPV status demonstrated a greater expression of chemokines in stromal tonsillar fibroblasts through IL1/IL1R1 signalling for the growth of tumours. These findings also support the progressive involvement of members of the IL1 signalling family in tumour development (46).

2.3.2 IL-1 BIOGENESIS

IL1R1 is the known agonist receptor in the IL1 family that triggers the activation of IL-1 ligand-mediated signalling. While IL-1 α and IL-1 β ligands are capable of binding to the IL1R1, triggering and executing the subsequent chain of command, both ligands need to be biologically activated before executing their biological function. Such activation is often accomplished by enzymes known to cleave and trim the immature ligands to their respective active form (47).

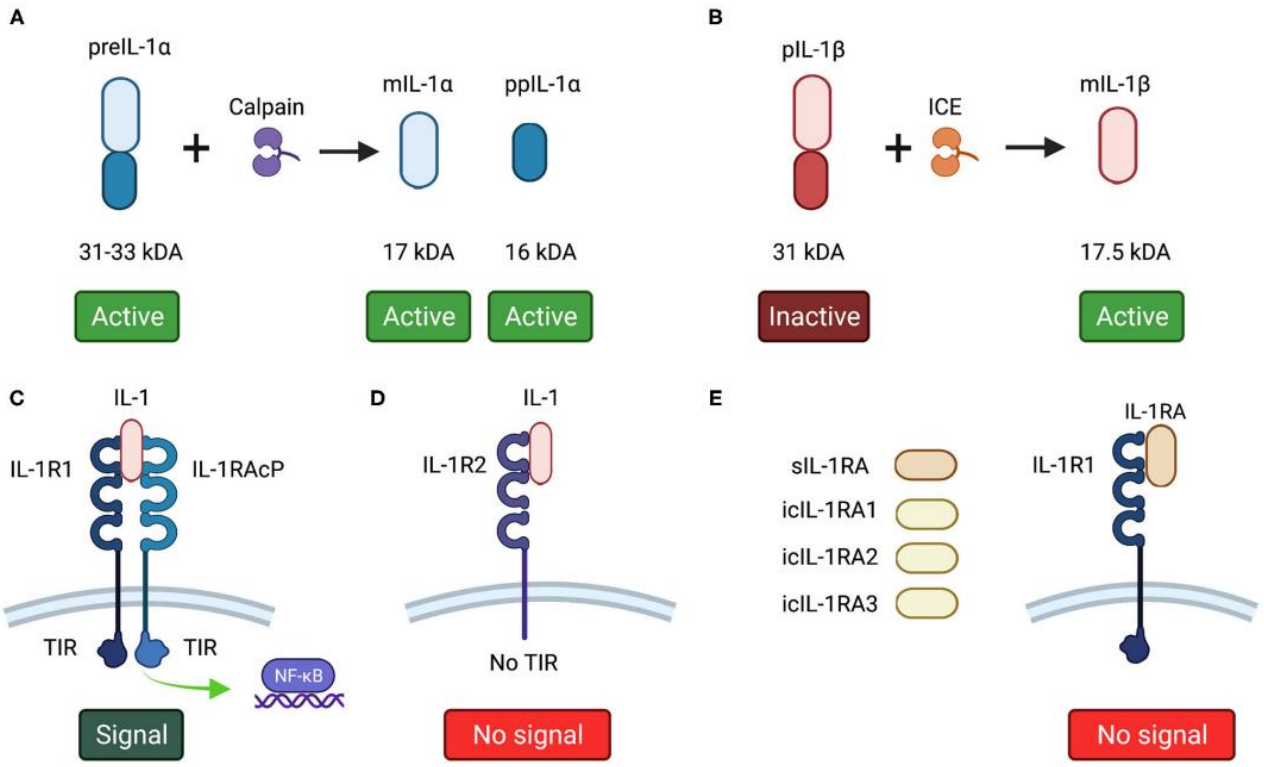


Fig: Biogenesis of IL-1 α *and IL-1* β. ***A****. The maturation of IL-1* α *is facilitated by the proteolytic cleavage of premature IL-1* α *by Calpain (a calcium-dependent cleavage enzyme).* ***B.*** *Maturation of IL-1* β *from its precursor form is achieved by the significant role played by ICE (IL-1 b Converting Enzyme).* ***C.*** *Activation of IL1R1 by IL-1 ligands facilitated by IL-1RAcP (accessory protein).* ***D.*** *IL1R2 as a decoy receptor diluting the biological activity of IL-1 ligands.* ***E.*** *Decoy ligand IL1RA binds to the IL1R1 and inhibits IL1/IL1R1 downstream signalling. Adapted from (51).*

IL-1 α is a well-known pro-inflammatory cytokine with pleiotropic activity. It is synthesized as a 31 kDa polypeptide in its precursor form cleaved and activated by Calpain, a calcium-dependent enzyme with proteolytic cleavage activity. The biological activity of calpain is triggered only under inflammatory conditions. Upon processing, the pre-IL-1 α is cleaved into a mature IL-1 α of 17kDa and 16kDa propiece with unknown function, however, some studies have suggested a role for intracellular pre-IL-1 α in regulating homeostatic function. The biologically active mIL-1 α is bound to the cell membrane with the capacity to activate juxtracrine-mediated cell signalling. IL-1 α signalling plays a major role in activating Nf-κB signalling, delaying apoptosis by inducing anti-apoptotic molecules. IL-1 α expression is often associated with the development and increase in invasive potential in Pancreatic Ductal Adenocarcinoma (PDAC), breast cancer and skin cancer (48).

IL-1 β, a powerful pro-inflammatory cytokine, is involved in many inflammatory reactions in tissues and organs. Pathological disorders such as diabetes and cancer are largely dependent on IL-

1 β signalling for their development, even though the exact mechanism of this signalling is still unknown. Immature pro-IL-1 β is proteolytically cleaved by ICE (IL-1 β Converting enzyme), which facilitates the biological activation of IL1-β. Although the activation mechanism underlying IL-1 β secretion is more difficult to understand, several theories have been proposed in the past to pinpoint the precise synthesis and secretion process (49). Hereditary periodic familial syndromes have been found to exhibit overexpression of IL-1β (50).

2.3.3. IL-1 FAMILY IN HEAD AND NECK CANCER

As IL-1 α, IL-1 β, and IL1R1 overexpression are known to be strongly linked to the progression of Head and Neck Cancer, the importance of IL-1 signalling in Head and Neck Cancer is further indicated by IL-1RA's under expression in the regulation of IL-1 signalling (51).

IL-1 α

The higher level of IL-1α expression is strongly associated with an increased risk of influencing distant metastasis. Such a nexus poses a grave threat with a leading cause of metastasis-derived death in Head and Neck Cancer. IL-1 α plays a crucial role in the regulation of major players like MMP, VEGF, IL-6, and IL-8 to be master regulators in facilitating metastasis. An increase in circulating IL-1 α can be considered to be a potential biomarker and helps to predict the severity of the disease's spread (52). Moreover, IL-1 α known to trigger the expression of another pro-inflammatory signaling cascade further aiding the tumour spread. It is shown that IL-1 α alongside IL-6, IL-8, and GM-CSF is capable enough to trigger the Nf-kB signaling that is capable of transcribing many cytokine genes (53). Alongside Nf-kB signalling, induction of AP-1 expression by IL-1 α increases the expression of Bcl-2, an antiapoptotic gene. The positive nexus between AP-1 and increased Bcl-2 mRNA transcripts explains the resistance towards apoptosis in Head and Neck Cancer tumour cells (54).

IL-1 β

Oral tumourigenesis is mainly reliant on IL-1 β regulation of the tumour microenvironment. High throughput bioinformatic analyses have determined that IL-1 β is essential in embarking the conditions for pro-tumourigenic activity in oral carcinogenesis (55). Though Tumour-Associated Macrophages (TAM) have a considerable impact on carcinogenesis, it is unclear how TAM contributes to the development of oral cancer, even though TAM is known to be able to secrete IL-1 β (56). IL-1 β is known for its aggressiveness in facilitating angiogenesis by activating the pro-

tumourigenic signaling cascade in oral cancer which was further ascertained in subsequent in vivo models. Altogether, the pathogenic role played by IL-1 β in the effect of oral carcinogenesis and metastasis and proves to be an efficient target (57).

IL1R1

The development of nasopharyngeal carcinoma and thyroid cancer is strongly correlated with polymorphisms in IL1R1, which are essential for the emergence of cancer. It has been discovered that the single nucleotide polymorphism rs3917225 of IL1R1 is highly associated with a number of diseases (58). It has been observed that IL-1 β activates the overexpression of IL1R1, which in turn facilitates the activation of the CXCR4 pathway. Tongue carcinoma has been linked to this overexpression of IL1R1 (59). The regulation of IL-1/IL1R1 mediated signaling is greatly influenced by HPV status, indicating that HPV-negative cells exhibit a greater degree of IL-1/IL1R1 mediated signaling than HPV-positive cells (46).

IL-1RA

The biological activity of IL-1/IL1R1 is tightly regulated by the competitive activity of IL-1RA, an antagonist to IL-1 ligands, thus diluting the signaling potential. Such antagonists act as a brake mechanism to evenly regulate and prevent the hijacking of the mechanism. Statistical data reveal an increase in the load of IL-1 ligands and IL1R1 in HNSCC patients compared to normal samples (60). *Nicklander et al.*, in 2021 inferred the downregulation of IL-1RA to the possible development of cancer by loss of expression of IL-1RA in the early dysplastic stage leads to attaining cellular immortality thus leading to carcinogenesis and subsequent activation of Nf-kB and subsequent signaling cascades. With no regulatory mechanisms in place, the deregulation of IL-1RA thereby facilitates the unchecked activation of pro-inflammatory cytokines. As a result of this unchecked activation, IL-6 and IL-8 are subsequently activated in cases of oral cancer, which is indicative of an inflammatory driven tumour progression (61).

2.4 TREATMENT FOR HEAD AND NECK CANCER

Surgery and chemo-radiation are often considered choices and gold standard approaches to treat locally advanced Head-Neck Cancer lesions. Such approaches often fail to have long-term positive impacts on patients owing to either underestimation of the tumour or failure to locate the origin of the tumour site (62). There is a growing need to find strategies that efficiently counter conventional treatment failure by identifying and targeting factors that are deemed to be crucial in enhancing the overall therapeutic results. Gene-based therapy is one of the considered strategies to improve overall treatment-related clinical output. Incorporating gene transfer-based therapy, and oncolytic virus-based therapy has gained traction in recent years and such approaches have given promising results that can be further broadened in the near future (63).

The significance of EGFR-mediated cell signaling is crucial in maintaining normal cellular function and any aberrant or dysregulated activation of EGFR in HNSCC is a worrisome concern and such over-expressed EGFR is often coupled to the aggressive nature of the tumour (64). Studies have shown that radiation often induces the release of pro-inflammatory cytokines like TGF- α leading to the activation of EGFR-mediated signaling (65). Underlining the importance of EGFR concerning HNSCC progression, it is imperative to block and/or neutralize the receptor-mediated signaling. Drugs ranging from monoclonal antibodies, tyrosine kinase inhibitors, and other inhibitors have gained momentum in recent years for their impeccable role in blocking EGFR-mediated signaling and controlling the proliferation of tumours (64).

2.4.1 CETUXIMAB

A chimeric monoclonal antibody, Cetuximab is considered to be the prime drug of choice in treating locally advanced HNSCC. Cetuximab in combination with radiation often proves to be efficient marked by a qualitative Progression-Free Survival accompanied by an increase in Overall Survival in patients (66). FDA in 2011 gave its approval to treat final stage HNC with cetuximab. The biologically active ligands like TGF- α and EGF that bind to EGFR are prevented by cetuximab's binding to the ligand-binding extracellular domain of EGFR present in its extracellular membrane. Due to its high affinity in binding towards EGFR, it prevents the receptor dimerization and subsequent activation of tyrosine kinase-mediated phosphorylation of the downstream signaling molecules (67). Cetuximab not only impedes the binding of ligands to EGFR but also mediates Antibody-dependent

cellular cytotoxicity in tumour cells, meaning the binding of Cetuximab to tumour cells attracts Natural Killer cells facilitating the neutralization of tumour cells (68).

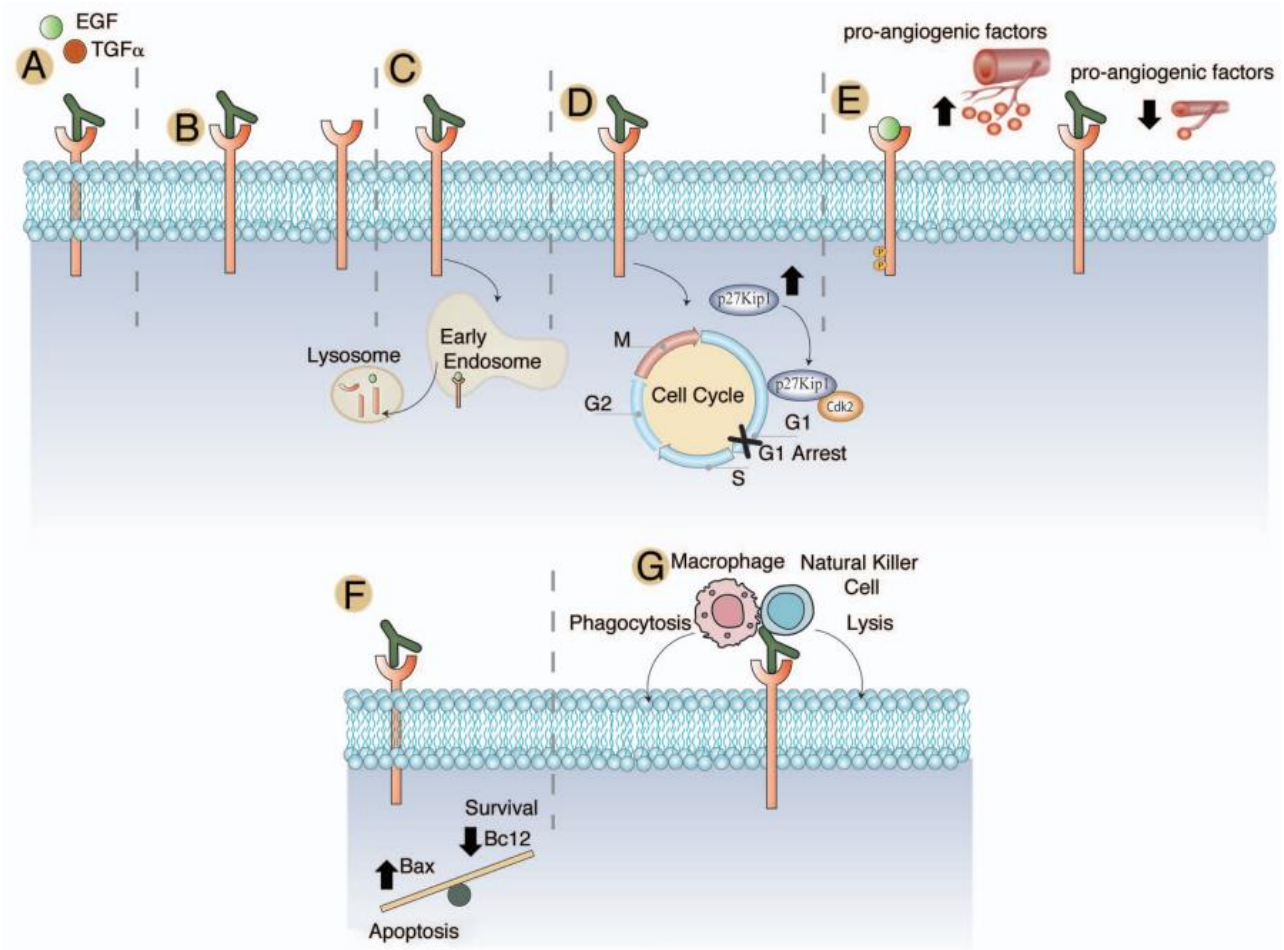


Fig: Cetuximab mode of action. Adapted from (69)

Cetuximab has a various biological function, including the suppression of cell cycle progression by stopping the cell cycle in adenocarcinoma cells at the G1 phase in human colon cancer. Additional research has demonstrated that Cetuximab functions as an antiproliferative agent in OSCC, as evidenced by a reduction in the activity of Cyclin-dependent kinase, an enzyme essential for promoting cellular proliferation. By suppressing CDK2 activity, which is indicated by an increase in p27, Cetuximab has an anti-proliferative effect on prostatic cancer (70).

2.4.2 CETUXIMAB - MEDIATED RESISTANCE

Drug resistance in HNSCC has numerous hypothesised mechanisms (71). One such mechanism of acquired resistance to chemo-therapeutic drugs that arises during the course of treatment is known as chemotherapeutic resistance. Accurately identifying the molecular mechanisms underneath the development of such resistance has become necessary since it represents a significant treatment barrier. Treatment regimens consisting of cisplatin, 5-fluorouracil, and docetaxel/paclitaxel drugs that are highly effective in fighting tumours can be linked to the development of such resistance, thus, in a global scenario, it is critical to identify the source of resistance and postulate counter-strategies (73).

There are several factors attributed to the development of resistance towards cetuximab. As Cetuximab is known to block angiogenesis, tumour resistance to Cetuximab is marked by an increase in angiogenesis. Studies have shown that tumours resistant to anti-EGFR blockers are marked with an increase in angiogenic activity (69). DiFi cells resistant to cetuximab showed a decrease in EGFR expression but an increase in the phosphorylated activity of Src kinase aiding cellular growth and survival was noted circumventing the activity of cetuximab (74). *Deric L.Wheeler et al.*, reported the Cetuximab-resistant cells showed an increase in expression of other ERBB family members namely HER2, HER3, and HER4 alongside the activation of other players namely c-Met thus, explaining the constitutive activation of signaling despite the negative role of Cetuximab on EGFR (75). Epithelial-Mesenchymal Transition accounts for Cetuximab resistance in Squamous Cell Carcinoma. Such correlation is marked by an increase in the expression of mesenchymal markers. Another mechanism towards Cetuximab resistance is the over-expression of EGFR which increases the ligand-independent dimerization of receptors and subsequent activation of downstream signaling, thanks to the overexpression of EGFR (76).

2.5 IL-1 THERAPEUTICS BLOCKING IL-1 SIGNALING

Given the diverse roles that the inflammatory cytokine IL-1 plays and its close relationship to a broad range of pathological conditions, it is critical to identify and comprehend strategies for reducing the pathological associations that are induced by IL-1 signaling. Biological agents are designed to inhibit/ neutralize these associations ranging from blocking the synthesis of IL-1 mRNA transcripts at the transcription level, blocking the processing of IL-1 ligands and receptor facilitating molecular signaling, inhibiting the secretion of the ligands, neutralizing the secreted ligand and receptor by directing monoclonal antibodies against them. Other strategies may include blocking the binding potential of IL-1 ligands to the receptor and blocking the downstream signaling molecules activated by IL-1 receptor activation (77). The notion of IL-1 neutralization aided with evidence shown to have a conclusive action in reducing the metastatic potential by downsizing the angiogenic capacity of tumours by neutralizing the biological activity triggered by IL-1 β (78). Suppressing IL-1 α activity in tumours has been shown to prevent tumour growth and has noted detrimental effects on angiogenesis (79).

2.5.1 XILOINX (BERMEKIMAB)

The human monoclonal antibody Bermekimab (MaBp1) has been developed to specifically target free IL-1 α ligand, synthesized from human B-lymphocytes immortalized by Epstein-Barr Virus (80,82). Studies have shown that there is no direct nexus between IL-1 neutralization and corresponding cytotoxic effects in tumour cells, yet it exposes a direct link between K-RAS positive tumours to IL1 α mediated signaling towards tumour progression (82). Patients suffering from colorectal cancer when treated with Bermekimab showed a substantial increase in Overall Survival, marked by an overall decrease in systemic inflammation, which is considered a crucial benefit in treating advanced colorectal cancer patients (83,85). Non-Small Cell Lung Cancer (NSCLC) patients treated with Xilonix showed a well-tolerated response marked by a significant increase in appetite and an overall decrease in pain and fatigue. Moreover, the efficacy of Xilonix was better in patients who were previously treated with anti-EGFR therapies. Such a combinatorial approach happens to be a strategy to improve cancer therapies (84).

Phase II clinical trials have demonstrated steady progress in significantly reducing inflammation-related lesions without any negative effects on drugs. Patients suffering from acne vulgaris and psoriasis showed an improvement in their medical condition when treated with Anti IL-

1 α antibody. However, patients suffering from Atopic Dermatitis showed little to no effect on the treatment with MaBp1 leading to the discontinuation of the phase II trial (81).

2.5.2 ANAKINRA (KINERET)

Pharmacologically, Anakinra alias Kineret is designed with the intention to target IL1R1 specifically. Anakinra functions as a ligand mimic for IL-1 α/β, binds to the biologically active IL1R1, and reduces the molecular signaling mediated by ligands and receptors. As rheumatoid arthritis is closely linked to chronic inflammation, Anakinra is one amongst the best drugs prescribed for treating patients with this condition and is thought to limit the biological response by inhibiting the pro-inflammatory response induced by IL-1 signaling (86). In regards to cancer therapy, induction of the anti-proliferative potential of Anakinra was tested in Multiple myeloma patients. Patients diagnosed with multiple myeloma were treated with Anakinra and found to be effective in diminishing the overall proliferative potential in the short term. However, Anakinra in addition to dexamethasone showed an increase in the overall biological effect exerted compared to Anakinra treatment alone, thus indicating Anakinra curtailed the proliferative potential of myeloma cells, while dexamethasone-induced apoptosis in myeloma cells (‘‘additive effect)’’ (87). The potency of Anakinra in depriving the metastatic potential of tumour cells is documented in bone metastatic breast cancer. *Ingunn Holen et al.*, asserted that Anakinra was effective in blocking the IL-1 β mediated signaling in bone metastatic breast cancer with a noted reduction in the overall development and progression of metastases. Anakinra exerted its pharmacological effect by blocking the proliferative potential of tumour cells in the bone region, thus prohibiting the metastatic potential (88). *Aditya Stanam et al.*, described that cells resistant to Erlotinib showed an uptick in IL-1 signaling activity and such resistance was overpowered by treating the cells with Anakinra. The Erlotinib-resistant cells when treated with Anakinra showed an increased growth inhibition which was attributed to the action of Anakinra in regulating the Tumour-Microenvironment (89). *Tong Wu et al.*, demonstrated Anakinra played a significant role in modulating genes responsible for the reprogramming of the Tumour Microenvironment. An *invivo* rat model with induced oral carcinogenesis with 4NQO (4-NitroQuinoline-1-Oxide), when treated with Anakinra, showed a decrease in the severity of the disease marked with histopathological evidence obtained from rat tongue (55).

2.6 CURRENT RESEARCH ON HEAD AND NECK CANCER

Several research studies have demonstrated the pivotal role that IL-1 plays in the advancement of tumours. From a therapeutic perspective, focusing on IL-1 signalling may enhance overall anti-tumour efficacy. However, addressing the target of IL-1 may encounter obstacles due to potential collateral damage-related concerns. The choice of a target, such as a ligand or a receptor, is a significant challenge since it can determine the overall effectiveness of treatment. The use of IL-1 neutralisation as a monotherapy or combinatorial therapy that targets the tumour microenvironment in addition to the tumour cells constitutes additional challenges. The major concern with anti-IL-1 therapy is the emergence of resistance to the treatment, which could further impede the improvement of patients who additionally correlate with a poor prognosis (90).

Previously in our laboratory, we reported that the combination of cetuximab, an anti-EGFR monoclonal antibody, and human-TRAP IL-1, a custom-designed monoclonal antibody, neutralises IL-1 α/β thus effectively inhibiting the proliferation of colorectal cancer cell lines that are resistant to cetuximab monotherapy. It can be inferred that tumour cell lines resistant to traditional anti-EGFR therapy namely cetuximab exhibited elevated levels of IL-1-mediated signalling, which is essentially non-existent in tumour cells sensitive to CTX. The CTX-resistant tumour cell lines displayed heightened sensitivity to the combinatorial treatment upon targeting both EGFR and IL-1/IL1R1 simultaneously, as evidenced by a reduction in the tumour cell lines overall proliferative capacity (91).

3. AIM

Mounting evidence depicts the role played by IL-1 signaling in aiding and abetting in overall tumorigenesis, yet their exact molecular mechanism/s pertaining to such actions are still under investigation. Targeting EGFR as the prime target as a treatment strategy often fails to benefit patients in the long run. Hence, it's imperative to amend the present treatment strategy by incorporating simultaneous targeting of EGFR accompanied by anti-IL-1/IL1R1 inhibitors to boost the overall efficacy of treatment-mediated prognosis. Thus, we categorized our work with the primary notion of deciphering the role of combinatorial drug action involving both anti-EGFR antibody and anti-IL-1/IL1R1 inhibitors in suppressing overall tumor proliferation. We aimed to decipher the induction of senescence potential by drugs in tumor cells and measure the aftermath of such effects in modulating tumorigenesis.

As IL-1 signaling exaggerates the development of tumor proliferation, we determined to discover the molecular signaling pattern/mechanisms provoked by IL-1 ligands by measuring critical parameters correlated to various signaling cascades. Finally, we are poised to measure the changes in various mechanobiological parameters that are critical for invasive and metastatic potential induced by combinatorial drug activity and their aftermath effects concerning tumor invasion and metastasis. Overall, this study design gives a glimpse into the simultaneous activity of both anti-EGFR antibody and anti IL-1/IL1R1 inhibitors concerning to amelioration of patients' agony with a clear determination of increase Progression-Free Survival and extended Overall Survival.

4. MATERIALS AND METHODS

4.1 CELLS, REAGENTS AND DRUGS

FaDu (HTB-43) cells, derived from hypopharyngeal tumor biopsy were purchased from ATCC. These cells are marked with wild-type EGFR, RAS, Akt which serves as an ideal vitro study model. The cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, USA) supplemented with 10% complement inactivated Foetal Bovine Serum (Thermo Fisher Scientific, USA), 1% L-Glutamine (Corning, USA) and 1% Penicillin and Streptomycin (Corning, USA). The cultured cells are kept in a humidified incubator with 5 percent CO2, which creates the perfect conditions for the cells. The cells were examined for the presence of mycoplasma, and mycoplasma positive cells were treated with BM cyclin (Roche Diagnostics, Germany) in accordance with the manufacturer's instructions.

Parallelly, FaDu CTX-R cells (resistant to cetuximab) were developed by constantly exposing the parental cells (sensitive to cetuximab) to cetuximab at an increasing concentration ranging from 100ng/mL to 20ug/mL (92). Once the acquired resistance of the cells was confirmed, the CTX-R cells were continuously maintained at 20ug/mL CTX.

- **Drugs and the respective concentration used in our study: -**

DRUGS	WORKING CONCENTRATION
1. Cetuximab (Erbitux) anti-EGFR antibody (CTX)	20ug/mL
2. Anakinra (Kineret) - anti-IL1R1 inhibitor (ANA)	50ug/mL
3. Xilonix (Bermekimab) anti IL-1 α antibody (XLX)	50ug/mL
4. IL-1 α/β ligands	10ng/mL each
5. h-TRAP IL-1 (anti IL-1 α/β custom-designed antibody) (TRAP)	20ug/mL

- The drugs are diluted in 1 X PBS and stored at $+4^0$C for a maximum period of 1 month.

4.2 PROLIFERATION ASSAY (ALAMAR ASSAY)

The analysis of the cell proliferative potential during treatment was conducted through the measurement of their relative absorbance. 1 X 10^3 cells were seeded with 100uL of full medium (FM 10%) in a 96-well as per the plate map and incubated overnight (to facilitate the adsorption of cells to the bottom of the plate). The following day, 20uL of Alamar blue dye (diluted at 1:500 in 1 X PBS) is added to each well and incubated in the dark for a period of 4 hours. Upon incubation, the relative absorbance was measured using a VICTOR ™ 1420 multilabel counter (Perkin Elmer, USA), at a wavelength of 595 nm. Initial absorbance was calculated as T0 values and the cells were treated with respective treatment, and incubated for 96 hours. The cells were again added with 20uL of Alamar blue dye to each well and incubated for 4 hours under the dark and the absorbance was measured and noted as T4. The relative absorption was calculated by measuring the difference between T4 and T0 values and analyzed for each treatment module in comparison to that of Control cells.

4.3 COLONY FORMATION ASSAY

2 X 10^3 cells were seeded in each well in a 24-well plate with 1000uL of FM 10% as per the plate map. The cells are initially incubated for a period of 24 hours facilitating the cells to well-adsorb to the bottom of the plates. The next day, old media is removed, 1000uL of media and respective treatment are added to each designated well and incubated for a period of 7 days. Upon treatment period, old media is removed, cells are washed and fixed with 4% PFA. After fixation, the cells were stained with 0.5% crystal violet, and the colonies formed were pictured and quantified through ImageJ. The relative covered area was calculated for each treatment module by comparing them to the control.

4.4 REAL-TIME PCR

Real-time PCR analyses were performed for genes that are believed to be critical and influenced by the treatment. To analyse the relative expression levels of such critical genes, 2 X 10^5 of parental cells and CTX-R cells were seeded in 6 cm plates with full medium and incubated. Once settled, the cells are starved by removing the old medium and supplemented with 1% FBS-DMEM for 24 hours. After starvation, the cells are treated with respected treatments in 1% FBS-DMEM and incubated for 48 hours. After treatment, the cells are washed with 1 X PBS, and total RNA was extracted from each sample using Qiazol treatment (Qiagen, The Netherlands) as per

the manufacturer's protocol. Isolated total RNA was resuspended in 30uL of RNase-free water, quantified, and their purity levels were assessed using NanoDrop™ One (Thermo Fisher Scientific, USA).

Upon quantification and purity assessment, 1 ug of total RNA was reverse transcribed to cDNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, USA) as per manufacturer protocol. Finally, reverse-transcribed cDNA was subjected to Real-Time expression analyses. The Real-Time PCR was performed in a 96-well plate in a thermal cycler a Bio-Rad CFX96 (Bio-Rad Laboratories, Hercules, CA, USA) using the $2^{-\Delta\Delta Ct}$ method. The B2M, α tubulin and β actin genes was used as internal control genes. Each sample was analysed in technical duplicates.

4.5 PROTEIN EXPRESSION ANALYSIS BY WESTERN BLOT

Expressed protein levels were validated by performing a Western blot. 1 X 10^5 cells were seeded in a 6cm plate with a full medium and incubated for 24 hours. The following day, the old media is removed and the cells are starved with 1% FBS-DMEM for 24 hours. The following day, the cells were treated with respective treatment regimens in 1% FBS-DMEM and incubated for 96 hours. Once treated, the cells are washed with 1 X PBS and the cells are collected by adding RIPA buffer supplemented with 1:100 PI and 1:100 Na_3VO_4. Isolated samples are transferred into 1.5mL Eppendorf tubes and the cells are mechanically disturbed by vortexing and centrifuged at 12800 rcf for 20 mins. Upon centrifugation, the supernatant is collected into new 1.5mL Eppendorf tubes. The collected total proteins were subjected to quantification and running samples were prepared accordingly.

Protein samples were prepared by adding 2 X Loading buffer (60 mM Tris-HCl pH 6,8; 5% glycerol; 0,2% SDS; 0,1 M dithiothreitol; 0,03 mM bromophenol blue), incubated at 100^0C for period of 10 mins (for sample activation). Prepared samples were loaded in 8% SDS gels and the total proteins were resolved by gel electrophoresis. Once resolved, the proteins were transferred into an

activated nitrocellulose membrane and blocked with 5% BSA. Upon blocking, the membranes were incubated with the primary antibody of our interest overnight at $+4^0$C. After incubation, the membranes were washed, and incubated with secondary antibodies for 1 hour, the protein bands were detected in the ChemiDoc XRS+ system (Bio-Rad, USA) treating the membranes with 1:1 WESTAR ECL substrates prior to detection. (Cyanagen, Italy). Expression levels of protein of interest were quantified using Image Lab software.

4.6 3-D SPHEROID ASSAY

3-D spheroid assay depicts quasi in-vivo model and helps to better understand the action of drugs in a 3-D environment. Prior to cell seeding, the wells of the plates were coated with 1mL of 2% autoclaved agarose gel to escape cellular adhesion (to provide a suspension environment). 2 X 10^5 cells were seeded in each pre-coated well as per the plate map along with 1 mL full media and respective treatments. The seeded cells were incubated at 37^0C for a period of 12 days and pictures of spheroids were taken at 4 X magnification. The captured pictures were processed in ImageJ software followed by measuring the volume of spheroids using ReVisp (a special software designed to calculate the total volume of spheroids using 2-D image configuration). Thus, the total volume of spheroids was measured in terms of Voxels (unit of spheroid volume measurement).

4.7 h-TRAP SYNTHESIS

1 X 10^6 of h TRAP IL-1 transfected cells were cultured in T-75 flasks in 10% FBS-DMEM. Once the cells confluency reaches 70-80%, the cells are sub-cultured at 8% FBS-DMEM, and the process is repeated until the percentage of FBS reaches 2%. Then, the cells are transferred to CD293 medium (CD293 medium for Suspension Cultures by Gibco Cat. No. 11913-019) a specialized medium for the culturing of transfected HEK293T cells with 2% FBS. Then, the cells are transferred into 25cm plates with 0% FBS-CD293 medium until the cell confluence reaches 100% (the cells confluence is noted in terms of medium turbidity as the absence of FBS inhibits the cells to adsorb onto the surface of the culture plates). Upon reaching the required confluence, the medium along with the cells are collected and centrifuged at 10000 rpm for 10 minutes at $+4^0$C. The supernatant is collected and filtered with 0.45 um and subjected to h TRAP IL-1 isolation. The h TRAP IL-1 are isolated using custom-designed beads that exhibit higher affinity towards h-TRAP IL-1 (affinity chromatography technique). The affixed h-TRAP IL-1 towards the beads are washed using 20 mM

sodium phosphate buffer (pH 7) eluted using 0.1 M glycine solution (pH 2.7). Isolated h-TRAP IL-1 are quantified by Lowry assay and Western blot for their purity and stored at -20 C.

The entire h-TRAP IL-1 isolation was performed using Protein G Sepharose™ 4 Fast Flow kit as per the manufacturer's protocol.

4.8 SENESCENCE STAINING

The senescence staining was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, USA) as per manufacturer protocol. Cells are seeded in a 96-well plate incubated overnight to facilitate the adsorption of cells. The following day, the old medium is removed, and 100uL of 10% FBS-DMEM along with necessary treatment is added and incubated. Once treated, the old medium is removed, and the cells are washed with 1 X PBS twice. Following, the cells are fixed with 1 X Fixative solution (diluted from 10 X fixative solution supplied with the kit in distilled water) for 15 minutes. The fixed cells are washed with 1 X PBS and stained with the prepared β-Galactosidase Staining Solution for overnight. The plates are sealed with parafilm and incubated at dark without CO_2. The following day, the cells are checked for any positive stain in positive control and the staining is stopped by removing the staining solution and the cells are pictured at 200 X magnification. Once pictured, the cells are overlaid with 70% glycerol and stored at + 4^0C.

4.9 IMMUNOHISTOCHEMISTRY

Both FaDu parental cells and CTX-R cells are seeded in 24-well pre-coated agarose gel wells at 2 X 10^6 per well. The cells are added with 1mL of 10% FBS-DMEM and respective treatment and incubated for a period of 12 days. Following the treatment period, the spheroids formed are collected in 1.5mL Eppendorf tubes and fixed with 10% formalin. Following the fixation, the spheroids are embedded in parafilm, sectioned, and stained with Eosin-Haematoxylin. Following, the stained slides are imaged at 20 X and 40 X magnification, and the captured images are analyzed.

4.10 LIVECYTE

Real-Time imaging and analysis of metrics was performed using the Phasefocus Livecyte™ platform. 1 X 10^3 cells were seeded in a 96-well plate as per the plate map configuration and incubated overnight. The following day, the old medium is removed, 100uL of fresh medium with appropriate treatment is added to each well and the plate is incubated inside the Livecyte incubator supplemented with 5% CO_2 at 37^0C. Images were captured at 30-minute intervals for a period of 48 hours and the metrics were stored for analysis. In our experiment, we committed to calculate two broad parameters namely cellular morphology and cellular motility. Once completed, the raw data were analyzed for the following parameters as follows: -

1. The cellular sphericity depicts the resemblance of cells close to a sphere. The metrics obtained from raw data calculate the sphericity of the cells influenced by drug treatment by considering the Volume of a cell, calculating the Surface Area of a sphere with that volume, and dividing through by the actual surface area of the Feature.

$$Sph = (\pi^{1/3} * 6V^{2/3}) / \text{Sur}$$

2. Instantaneous Velocity is calculated as the change in position in frame (n) from frame (n-1) divided by time at frame (n) – time at frame (n–1).

3. Confinement ratio defines the method of movement of a cell. This parameter is calculated by considering the meandering index and multiplying the square root of track speed duration in hours. $CR = MI * \sqrt{T}$.

4.11 STATISTICAL ANALYSES

Statistical significance was evaluated using GraphPad software. Ahead of calculating statistical significance, all the raw data were processed for any outliers present, and the identified outliers were eliminated, thus only cleaned data were considered. The statistical significance was enumerated based on the following p-value: $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***; $p < 0.0001$ ****.

5. RESULTS

5.1 Over-expressed IL-1 α/β & IL1R1 correlate with decreased Overall Survival (OS) in regional and distant lymph node-positive patients.

The cytokine-mediated cell signaling plays a pivotal role in maintaining homeostasis, while deregulated expression of certain classes of cytokines proved critical in inflammation and progression of neoplasms. Such altered expression levels of pro-inflammatory cytokines namely IL-1, IL-6, IL-8, and TNF-α are found to play a significant role in determining the prediction and overall prognosis in patients (93). IL-1 signaling holds a critical sway in inflammation-related tumour progression, therefore it is imperative to interpret the exact role of IL-1/IL1R1 signaling nexus in neoplasm (94). Such molecular setup has increased the necessity of studying the role of IL-1 and IL1R1 signaling in tumour progression and prediction of prognosis in Head and Neck Cancer. By delineating the exact role of the IL-1/IL1R1 signaling axis in Head and Neck Cancer, we aim to improvise and modulate existing treatment strategies and extend the qualitative Overall Survival (OS) rate in patients. To support our claim, we analysed RNA-Seq data derived from tumour patients of Head and Neck Cancer depicting the expression levels of IL-1 α/β & IL1R1 and categorized into three different metastatic stages namely negative metastatic, regional lymph node metastatic, and distant metastatic modes and their corresponding OS rates.

The comprehensive data analyses showed that the amount of expression of IL-1 α/β & IL1R1 has a strong correlation in determining the OS rate in patients. In detail, figure 1A depicts the patients with negative metastatic lesions showed no difference in Overall Survival when stratified according to higher/lower expression levels of IL-1 α/β & and IL1R1 respectively, thus excluding their role concerning tumour progression. On the other hand, differential expression levels of IL-1 α/β & IL1R1 play a crucial role in determining the Overall Survival rate in regional and distant lymph node metastatic patients. Patients harbouring regional metastatic tumour lesions showed a considerable yet, not statistically significant decrease in Overall Survival rate corresponding to high levels of IL-1 α/β. No trend was instead observed in the same category when IL1R1 was analysed.

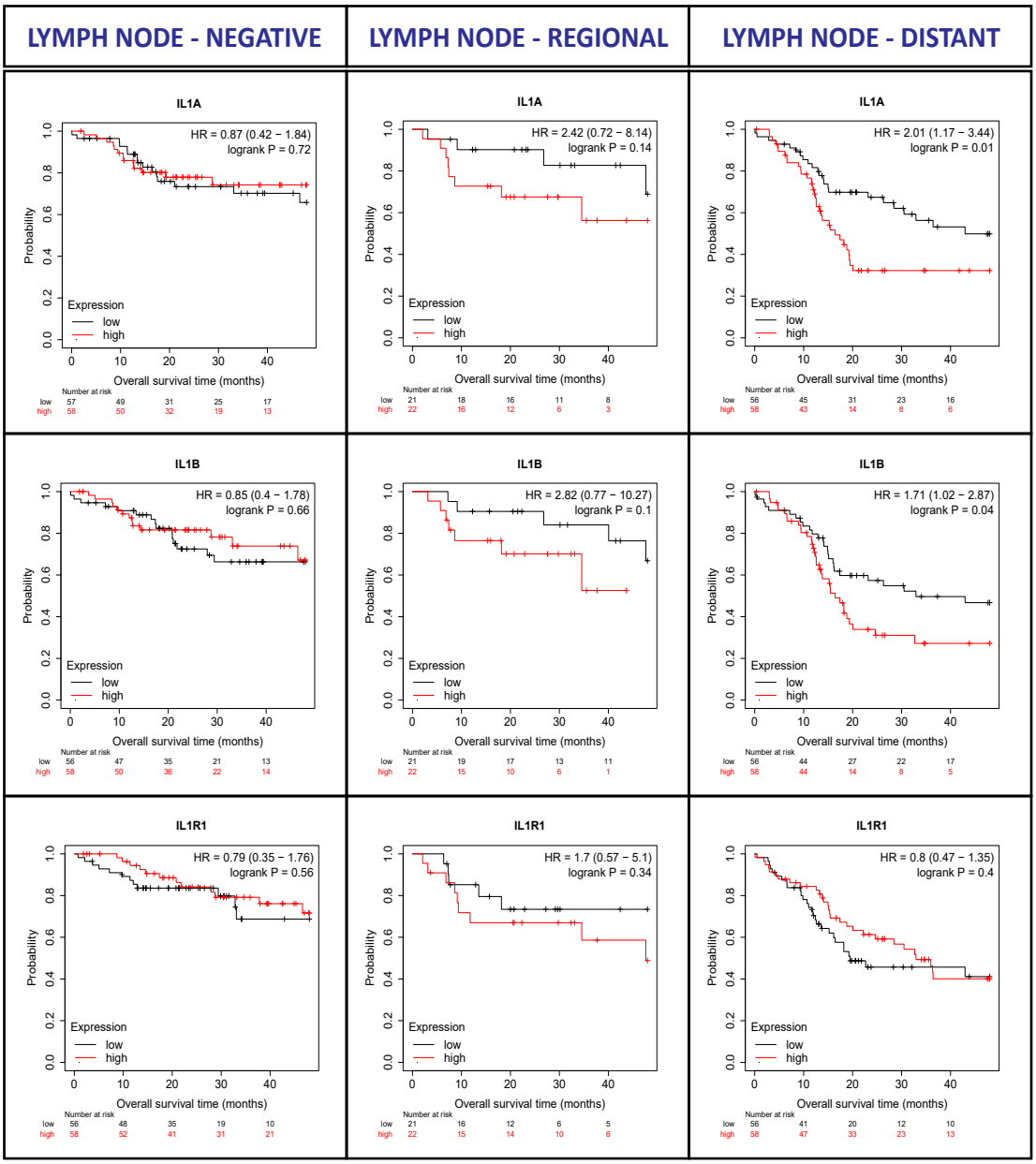
Strikingly, by following the trend of the whole dataset in patients harbouring distant metastatic tumour lesions, a significant decrease in Overall Survival rate was correlated to higher expression levels of IL-1 ligands namely IL-1 α and IL-1 β. Here, increasing amounts of IL-1 α and

IL-1 β severely impact tumour patients survival compared to lower expression levels of these ligands. Of note, no such difference with respect to the expression level of IL1R1 corresponding to survival rate is noted in distant metastatic patients.

Upon comprehensive analyses of the dataset, we corroborate a strong correlation between the IL-1 α/β & IL1R1 signaling and its corresponding Overall Survival rate in regional and distant lymph node metastatic patients. Figure 1C depicts the dataset that includes all parameters like race, sex, age, and other domains within its scope to better understand the modus of action of the IL-1 α/β & IL1R1 signaling at different metastatic stages in tumour progression and lays the foundation to demonstrate the role of IL-1 signaling and further exploring ways to increase the treatment efficacy in patients.

FIGURE 1

A



B

Gene expression	Level of expression	Negative metastasis	Regional metastasis	Distant metastasis
IL-1 α	Low	n=179	n=74	n=171
	High	n=172	n=59	n=129
IL-1 β	Low	n=174	n=71	n=166
	High	n=180	n=54	n=129
IL1R1	Low	n=168	n=60	n=139
	High	n=203	n=67	n=174

C

STATUS	ALL
RACE	ALL
AJCC N	ALL
AJCC T	ALL
STAGE	ALL MARGIN
GENDER	ALL
GRADE	ALL
SMOKING HISTORY	ALL
ALCOHOL HISTORY	ALL

FIGURE LEGEND:

Fig: 1. Expression analyses of IL-1 α/β and IL1R1 at various metastatic stages.

A. *The prediction of Overall survival (OS) in Head and Neck Cancer (HNC) patients are directly proportional to expression levels of IL-1 ligands and receptor. The Kaplan-Meier plotter depicts a comprehensive decrease in OS rate in patients exhibiting higher expression levels of IL-1 ligands and receptor compared to patients with low expression levels. Such a distinct difference in OS is quite evident in patients harbouring regional and distant neoplasm. On the contrary, non-metastatic patients showed no difference in relative expression levels of ligands and receptor affecting their OS, thus portraying the role of IL-1 and IL1R1 as silent spectators. In support of the notion, it is obvious that a tight signaling nexus does exist between IL-1 signaling and OS in HNC patients.*

B. *Categorization of patients dataset into three distinct cohorts namely non-metastatic, regional metastatic, and distant metastatic patients, and the corresponding number of patients assessed in each cohort. A total of 2369 patient data were assessed for expression analyses. The dataset were broadly classified into three distinct groups namely negative metastatic, regional lymph node metastatic, and distant lymph node metastatic groups with each group sub-grouped based on higher or lower expression levels of the concerned parameters.*

C. *Kaplan-Meier plotter depicts complied data derived from tumor patients that included the holistic assessment of various parameters as mentioned.*

5.2 Development of Cetuximab-resistant (CTX-R) cell line

Head and Neck Cancer Squamous Cell Carcinoma (HNSCC) is marked with overexpressed EGFR, thus making it a principal target in treating Head and Neck Cancer. As the neoplasm is derived from the epithelial cellular origin (carcinoma), its dependency on Epidermal Growth Factor Receptor-mediated signaling for their survival becomes inevitable for targeting and treating the tumour (95). Cetuximab, a chimeric monoclonal antibody (FDA-approved 2006) is the primary drug combined with other chemotherapeutic drugs to treat metastatic colorectal cancer (96) and further expanded to treat Head and Neck cancer. Due to its increased affinity towards extracellular EGFR, cetuximab is often combined with radiation to treat locally advanced Head and Neck neoplasms. Patients treated with cetuximab + radiation initially showed some good prognosis, yet, continuous exposure of tumour to Cetuximab led to the development of treatment-induced resistance or Cetuximab-induced resistance in patients thus complicating the treatment strategy correlated with poor prognosis.

We first developed Cetuximab-resistant (CTX-R) cells (figure 2A) by constantly exposing the FaDu cells to increasing concentrations of cetuximab ranging from 100ng/mL to 20ug/mL. To test whether the CTX-R cells indeed have gained resistance towards cetuximab, both parental sensitive cells and CTX-R cells were seeded in a 96-well plate, treated with and without CTX. We performed a viability assay by measuring the degree of resazurin substrate reduction in both parental cells and CTX-R cells. We detected an overall decrease in relative viability in parental cells treated with cetuximab while the CTX-R cells showed no inhibition when treated with and without cetuximab (figure 2B). This further confirmed that CTX-R cells indeed have attained resistance towards cetuximab.

Now, we asked whether the decrease in cellular proliferation was associated with an enhancement in senescence cells. Thus, we treated both parental cells and CTX-R cells and incubated them, the cells were fixed and stained for senescence activity in cells (β-Galactosidase Staining). The development of blue-coloured stained cells indicates positive senescent cells. Figure 2D illustrates the parental cells showed a significant increase in the number of positive senescent cells when treated with CTX compared to control cells. Apparently, CTX treatment induced a significant increase in the number of positive senescent cells which were counted as the number of β-Galactosidase stained cells/on the number of total cells counted. Of note, CTX-R cells showed very little senescent cells under cetuximab treatment, thus confirming the acquired resistance to the antibody.

Finally, we determined to decipher the activity of cetuximab in hindering the replication mechanism of cells by measuring the cell doubling time in both parental cells and CTX-R cells. FaDu parental cells when treated with cetuximab showed a three-fold increase in cell doubling time, compared to untreated cells suggesting the presence of CTX hampers cell proliferation thus, explaining the negative role of cetuximab in blocking EGFR signalling that is vital for epithelial cell proliferation (figure 2C). Strikingly, CTX-R cells showed a decrease in cell replication in the presence of cetuximab compared to cells without treatment (FM 10%) explaining that CTX-R cells have become addicted to CTX thus the absence of CTX slowed down their proliferation rate as evidenced. This is yet another piece of evidence to claim that CTX-R cells indeed gained resistance towards the negative role of cetuximab.

FIGURE 2

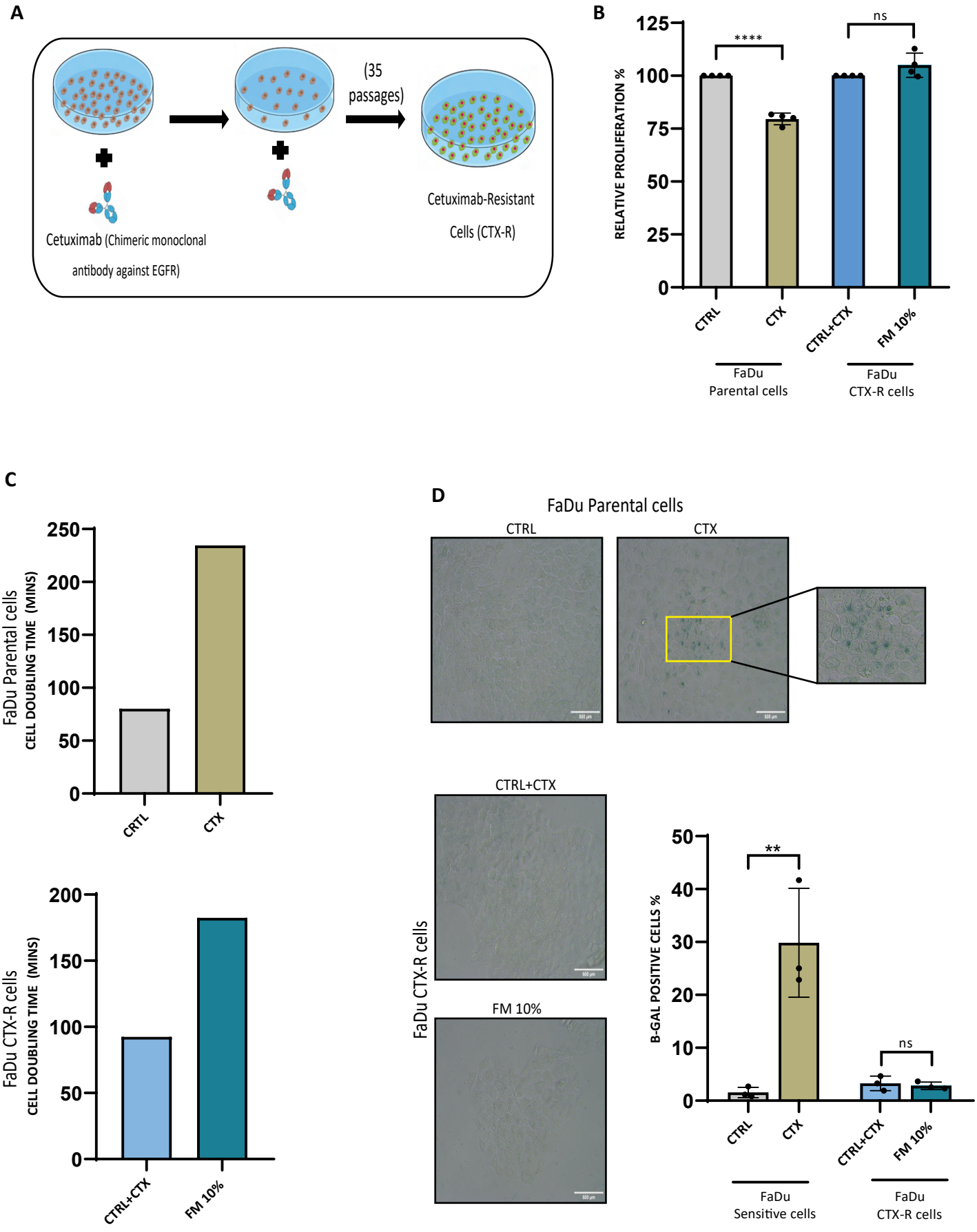

A
(35 passages)
Cetuximab-Resistant Cells (CTX-R)
Cetuximab (Chimeric monoclonal antibody against EGFR)
B
RELATIVE PROLIFERATION %
125
100
75
50
25
0
****
ns
CTRL
CTX
CTRL+CTX
FM 10%
FaDu Parental cells
FaDu CTX-R cells
C
FaDu Parental cells
CELL DOUBLING TIME (MINS)
250
200
150
100
50
0
CRTL
CTX
FaDu CTX-R cells
CELL DOUBLING TIME (MINS)
200
150
100
50
0
CTRL+CTX
FM 10%
D
FaDu Parental cells
CTRL
CTX
CTRL+CTX
FaDu CTX-R cells
FM 10%
B-GAL POSITIVE CELLS %
50
40
30
20
10
0
**
ns
CTRL
CTX
CTRL+CTX
FM 10%
FaDu Sensitive cells
FaDu CTX-R cells

FIGURE LEGEND

Fig: 2 Continuous exposure of parental cells to Cetuximab resulted in the emergence of CTX-R cells.

A. Development of CTX-R cells - *a schematic overview of the development of Cetuximab-resistant cells (CTX-R). The CTX-R cells developed from parental cells by being continuously cultured in the presence of cetuximab at an increasing concentration starting from 100ng/mL to 20ug/mL for 35 passages.*

B. Proliferation assay (Alamar assay) - *Relative proliferation of both parental cells and CTX-R cells was assessed by measuring their relative cell growth in the presence and absence of CTX. Parental cells showed inhibition in cell growth when treated with CTX (20ug/mL), while no inhibition with respect to cell proliferation is evident in CTX-R cells when treated with CTX (20ug/mL) thus proving CTX-R cells have gained resistance towards CTX. Statistical significance ****P < 0.0001 (Student t-test).*

C. Cell Doubling time – *Cell doubling time under the proliferation parameter is assessed in both parental cells and CTX-R cells. Parental cells showed a three-fold increase in cell doubling time compared to vehicle, while in CTX-R cells, cells treated with CTX (20ug/mL) showed a lower cell doubling time compared to cells treated without CTX, thus explaining the cetuximab hampers cellular replication machinery with a delay in cell replication time in parental cells, while, CTX-R cells are devoid of cetuximab action in presence of cetuximab.*

D. β-galactosidase assay – *Final validation on CTX-R cells for their acquired resistance towards CTX was performed by the identification and quantification of senescent positive cells by β-Galactosidase Staining assay. FaDu Parental cells and CTX-R cells are treated with and without CTX (20ug/mL) for a period of 6 days. The treated cells were fixed with 4% Paraformaldehyde stained with β-Galactosidase Staining and quantified based on counting the number of senescent positive cells marked with blue staining. Upon quantification, Parental cells treated with CTX (20ug/mL) showed an increased percentage of positive senescent cells compared to control cells with no cetuximab treatment, while CTX-R cells showed negligible to no positive senescent cells when treated with CTX (20ug/mL) and without CTX (FM 10%). Statistical significance **P < 0.05 (Student's t-test).*

5.3 - IL-1/IL1R1 abrogation overcomes drug sensitivity by downregulating the overall proliferation of cells

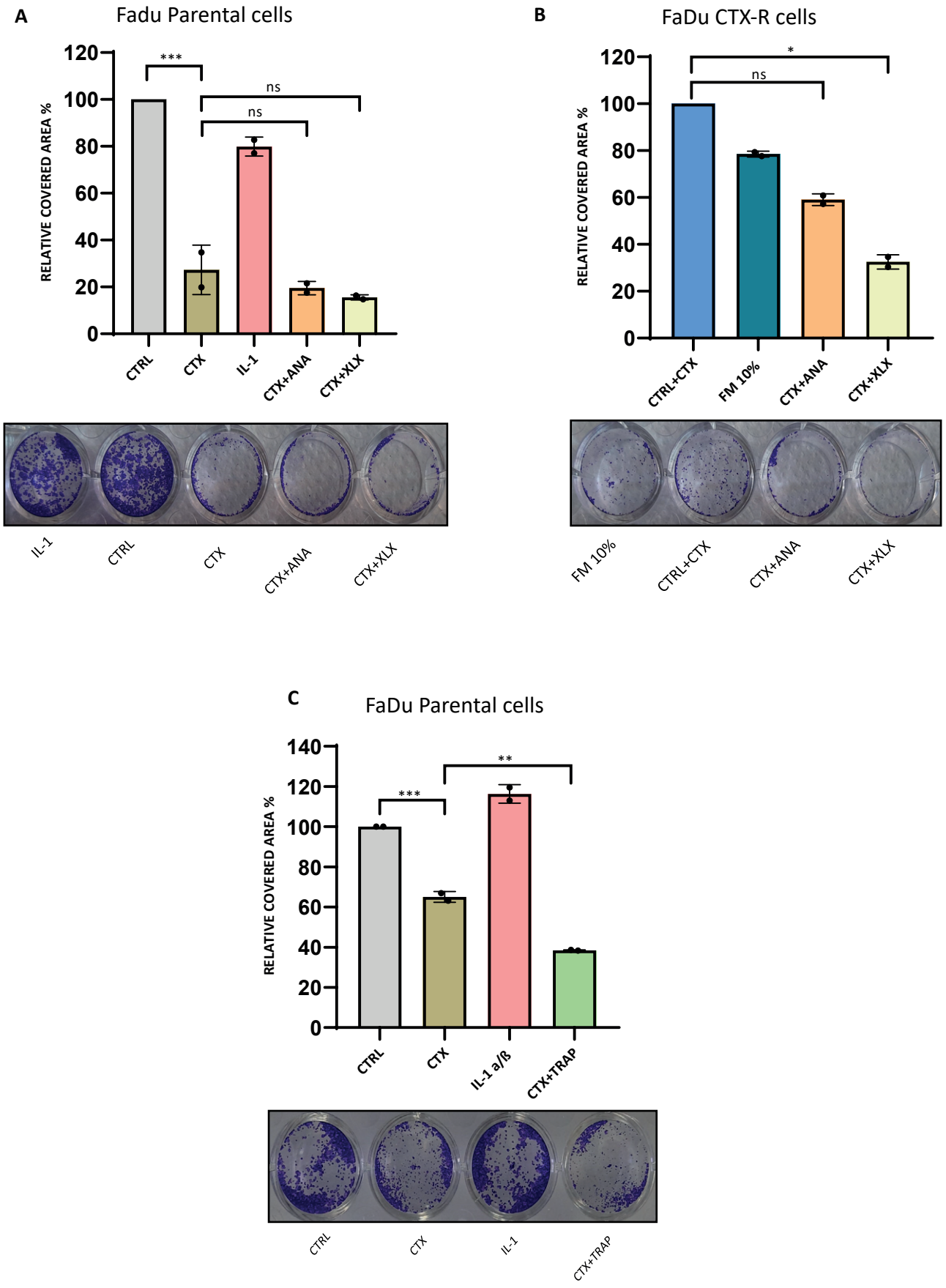
It is a well-established fact that IL-1 signaling plays a pathological role in both local and systemic inflammation (97) and inflammation promotes tumour expansion and progression in a network with tumour-microenvironment. In inflammation-associated/mediated tumour progression, targeting inflammation-inducing cytokines amplifies the chances of containing overall tumour progression (98). Our patient-derived data suggested that IL-1 signaling serves to be a master regulator in determining the OS in patients, hence, we determined to analyse the inhibition of the IL-1 signaling cascade by employing anti-IL-1/IL1R1 inhibitors alongside anti-EGFR inhibitors to check the combinatorial drug efficacy in downgrading overall cellular proliferation. By specifically targeting IL-1 α, IL-1 β, and IL1R1 trilogy alongside EGFR, we aimed to understand their aftermath effect in the overall containment of tumour proliferation on par with existing therapies. Thus, we employed two different inhibitors that specifically nullify the activity of IL-1 α and IL1R1 and determined to measure their combined efficacy alongside CTX. Xilonix (Bermekimab) is an anti-IL-1 α monoclonal antibody (decoy receptor) that specifically binds to free ligands of IL-1 α, thereby neutralizing the cytokine-triggered signaling that is vital for tumour progression (84). Anakinra (Kineret) is an anti-IL1R1 inhibitor that specifically binds to IL1R1 as a decoy ligand with no active biological duty thus obstructing the binding potential of biologically active IL-1 α/β ligands to the receptor and inhibiting the ligand-receptor mediated downstream signaling (99). These inhibitors can energetically target both IL-1 ligands and IL1R1 and could potentially increase the efficacy of an anti-EGFR inhibitor namely cetuximab in treating HNSCC. Cetuximab has a proven capability of downregulating the overall proliferation of cells by binding to the extracellular domain of EGFR thereby blocking the RAS and/or Akt/mTOR-mediated signaling pathways critical for cellular proliferation. Considering the disastrous role of IL-1 signaling in tumorigenesis, it becomes obvious to assess the improved efficacy of CTX treatment by simultaneously blocking IL-1 α/β & IL1R1 and EGFR thereby bolstering the overall efficacy of CTX (additive effect).

Employing IL-1 antagonists and anti-IL-1α neutralization in combination with cetuximab, we detected an improvement in the overall efficacy of cetuximab in both parental cells and CTX-R cells. In detail, both parental cells and CTX-R cells were seeded, incubated, and treated with drugs. Upon treatment, we measured the relative covered area for each treatment in comparison to the control cells. Figure 3A., Parental cells when treated with CTX showed a statistically significant decrease in the number of colonies formed, while parental cells when treated with both CTX+ANA and

CTX+XLX showed a subtle but statistically not significant decrease in the number of colonies compared to CTX treatment alone. On the other hand, (figure 3B) CTX-R cells when treated with XLX showed a statistical significance marked by a decrease in the total number of colonies formed compared to cells grown in the presence of CTX alone, while CTX-R cells treated with CTX+ANA showed a subtle yet statistically non-significant decrease in number of colonies formed.

Additionally, we determined to investigate the potential of our laboratory-designed IL-1 α/β inhibitor named h-TRAP IL-1. h-TRAP IL-1 (TRAP) is a custom-designed decoy receptor that was found to be effective in impeding IL-1 α/β ligands. To measure its efficacy on par with CTX, the FaDu parental cells were treated with CTX alone and in combination with CTX and TRAP. Cells treated in combination with TRAP and CTX proved to be more effective in bolstering the overall efficacy of Cetuximab by inhibiting cellular proliferation (figure 3C), signifying in reduction in the number of colonies formed compared to cells treated with CTX alone. These data strengthen the ideal aim of our hypothesis that co-targeting both IL-1/IL1R1 and EGFR boosts the degree of anti-EGFR activity in tumour cells.

FIGURE 3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Fadu Parental cells
RELATIVE COVERED AREA %
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IL-1
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IL-1
CTRL
CTX
CTX+ANA
CTX+XLX
B
FaDu CTX-R cells
RELATIVE COVERED AREA %
120
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CTRL+CTX
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CTX+ANA
CTX+XLX
C
FaDu Parental cells
RELATIVE COVERED AREA %
140
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20
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***
**
CTRL
CTX
IL-1 a/ß
CTX+TRAP
CTRL
CTX
IL-1
CTX+TRAP

FIGURE LEGEND

Fig: 3 Anti IL-1 & IL1R1 inhibitors alongside CTX downregulates overall cellular proliferation in both parental and CTX-R cells.

- ***Colony formation assay in Parental cells (A) and CTX-R cells (B) –*** *CTX in combination with ANA and XLX is proven to be effective in downregulating overall cellular proliferation compared to CTX (20ug/mL) alone. Both parental cells and CTX-R cells were seeded at 2.000 cells per well in 24-well plates and treated with CTX (20ug/mL) alone, CTX (20ug/mL) + ANA (50ug/mL), and, CTX (20ug/mL) + XLX (50ug/mL) along with CTRL and incubated for 7 days. Upon treatment, the cells were fixed and stained with 0.5% crystal violet and quantified by counting the number of colonies formed. Upon quantification of number of colonies formed, a decline in the total number of colonies formed was evident in both parental cells and CTX-R cells when treated with both anti-EGFR and anti-IL-1* α inhibitors *compared to CTX alone. Statistical significance ***P < 0.001, *P < 0.05.*

- ***C.*** *h-TRAP IL-1 showed increased effectiveness in reducing overall colonies formed when parental cells were treated with TRAP (20ug/mL) and CTX (20ug/mL) compared to CTX (20ug/mL) treatment alone. Statistical significance **P < 0.01.*

5.4 IL-1 antagonists increase CTX-mediated cellular senescence in parental cells

Senescence is a cellular phenomenon where the normal cells lose their ability to actively replicate yet, retain certain metabolic activities. (100). Such senescence is triggered by diverse factors ranging from the shortening of telomeres, oxidative stress, and DNA damage, and such behaviour of cells is often characterized by an increase in the expression of the Senescence-associated Secretory Phenotype (SASP) ultimately driving to cell death (101). Cancer cells that undergo senescence can be considered beneficial due to their loss of replicative potential and this principle of treatment-induced senescence can be readily employed in treating cancer.

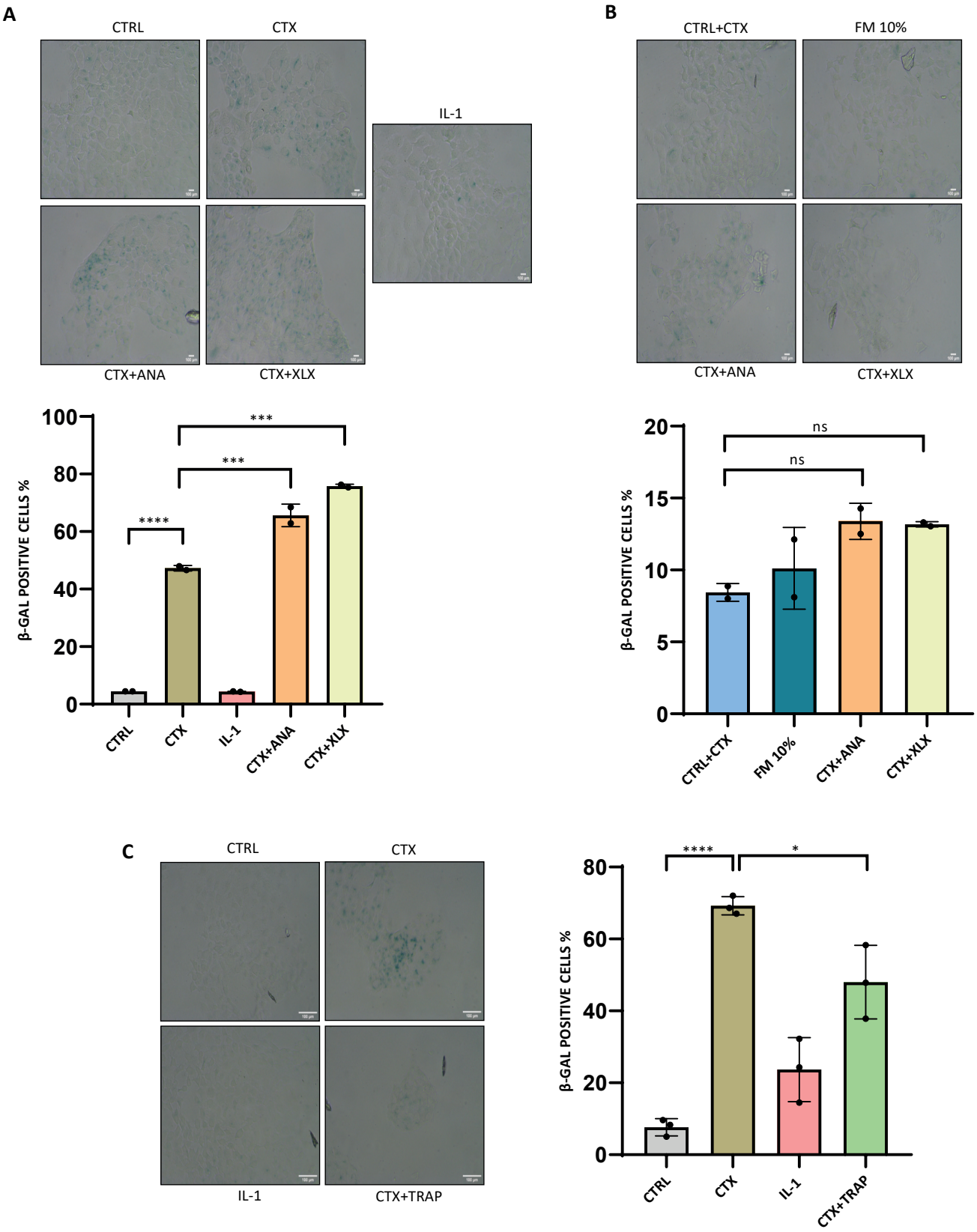
Therapy-mediated senescence or treatment-induced senescence are quite successful in abating overall tumour load. To validate the chemotherapeutic-induced senescence potential of anti-IL-1/IL1R1 inhibitors, both parental cells and CTX-R cells are seeded in a 96-well plate as biological triplicates and treated with Cetuximab, Cetuximab + Anakinra, and Cetuximab + Xilionix. Subsequently, the treated cells were fixed with 4% paraformaldehyde, stained with β-Gal, and imaged and enumerated for positive senescent cells. FaDu parental cells treated with Cetuximab and Anakinra, Cetuximab, and Xilonix showed an increased senescence activity marked with positive stained senescent cells compared to cells treated with Cetuximab alone (figure 4A), thus explaining the combined potential of both anti-EGFR and anti-IL-1/IL1R1 inhibitors. Surprisingly, such a noted effect in parental cells failed to reflect in CTX-R cells, meaning the treatment failed to induce senescence in CTX-R cells (figure 4B).

- **h-TRAP IL-1 is found effective in inducing replicative senescence in FaDu parental cells**

Replicative senescence is a molecular mechanism through which cells undergo cell cycle arrest marked by curtailment of the telomeric region thereby reducing the overall proliferation of cells (102). The shortening of lagging DNA strands during replication triggers numerous factors responsible for cell-cycle arrest and cell death. Cancer cells evade replicative senescence and achieve immortality, thanks to the activity of hTERT (human Telomerase Reverse Transcriptase), an enzyme-oligonucleotide factor critical in extending the lengths of long strands of DNA. We strived to assess the disruptive nature of h TRAP IL-1 in diminishing the activity of hTERT in cancer cells.

We hypothesize that the activity of h-TRAP IL-1 in combination with Cetuximab is capable of inducing replicative senescence in parental cells. FaDu parental cells were seeded in a 96-well plate and treated with CTX and CTX+TRAP for a period of 144 hours. The cells were fixed and stained with a β-Gal staining. Upon analysis, cells treated with CTX alone showed an increased percentage of senescent-positive cells compared to cells treated with the combination of CTX+TRAP indicating the combination of drugs failed to induce senescence in cells (figure 4C). However, we also found that the cells treated with CTX+TRAP showed an overall decrease in the total number of cells with a low percentage of senescent positive cells, thus hypothesizing that h-TRAP IL-1 may be involved in curtailing replicative potential of cells with a poor effect in inducing true senescence (thus giving a different idea on modus of action of h-TRAP IL-1 compared to the action of other drugs namely Anakinra and Xilonix). This implies that the drug combination of CTX+TRAP may induce chemotherapy-mediated replicative inhibition at the cell cycle level. This paves the way to further understanding the exact role of human TRAP IL-1 in treating cancer patients.

FIGURE 4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CTX
IL-1
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CTX+XLX
β-GAL POSITIVE CELLS %
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CTX+ANA
CTX+XLX
β-GAL POSITIVE CELLS %
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IL-1
CTX+TRAP
β-GAL POSITIVE CELLS %
80
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0
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*
CTRL
CTX
IL-1
CTX+TRAP

FIGURE LEGENDS

Fig: 4 - Anti-IL-1/IL1R1 alongside anti-EGFR facilitates an increased senescence activity in parental cells.

- ***A & B***. *Both parental cells and CTX-R cells were seeded and treated with CTX, CTX + ANA, and CTX + XLX. Treated cells were fixed with 4% PFA, followed by staining with β-Gal stain, and incubated overnight. Positively stained cells are marked with the presence of a blue stain. Upon enumeration of the number of positive cells, parental cells showed an increased senescent activity marked by an increase in the number of positively stained cells treated with CTX (20ug/mL) +ANA (50ug/ml), and CTX (20ug/ml) + XLX (50ug/mL) compared to CTX (20ug/mL) alone. On the contrary, no such significant increase in senescence activity was reported in CTX-R cells.*

- ***C.*** *Combinatorial activity of h TRAP IL-1 alongside CTX proved effective in curtailing cellular replication in parental cells. Parental cells were seeded and treated with CTX and a combination of CTX + TRAP. After the treatment period, the cells are fixed with 4% PFA, stained with β-Gal staining, and evaluated for positive senescent cells. Cells treated with CTX (20ug/mL) showed an increase in senescent activity with a larger proportion of positive cells while cells treated with the combination of TRAP (20ug/mL) and CTX (20ug/mL) showed a decrease in the overall cellular population and presence of significant positive senescent cells. This explains the notion that the combination of CTX+TRAP has an antagonistic effect in curtailing cellular proliferation.*

5.5 Anti EGFR and anti-IL-1 combination increases IL1R1 expression along with Hp1γ positivity in parental cells.

As previously reported, the combination of anti-EGFR and anti-IL-1/IL1R1 inhibitors in the treatment module increases the capability of downregulating cellular proliferation by inducing senescence-related arrest in parental cells. We sought to further confirm these data, thus first we evaluated the expression levels of IL1R1 upon treatment with CTX and combination with antiIL1 inhibitors. Through Real-Time PCR analyses, we found that a subtle increase in the relative expression of IL1R1 was observed in parental cells (figure 5C) when treated with anti-EGFR and anti-IL-1α compared to cells treated only with CTX. The same level of expression of IL1R1 was observed in CTX-R cells (figure 5F) when treated with the combination of anti-EGFR and anti-IL-1α compared to cells exclusively treated with anti-EGFR. These data suggested that anti-EGFR re-routes the signaling towards IL-1 axis activation, as detected with the increased expression of IL1R1, in both parental cells and CTX-R cells, under the burden of treatment.

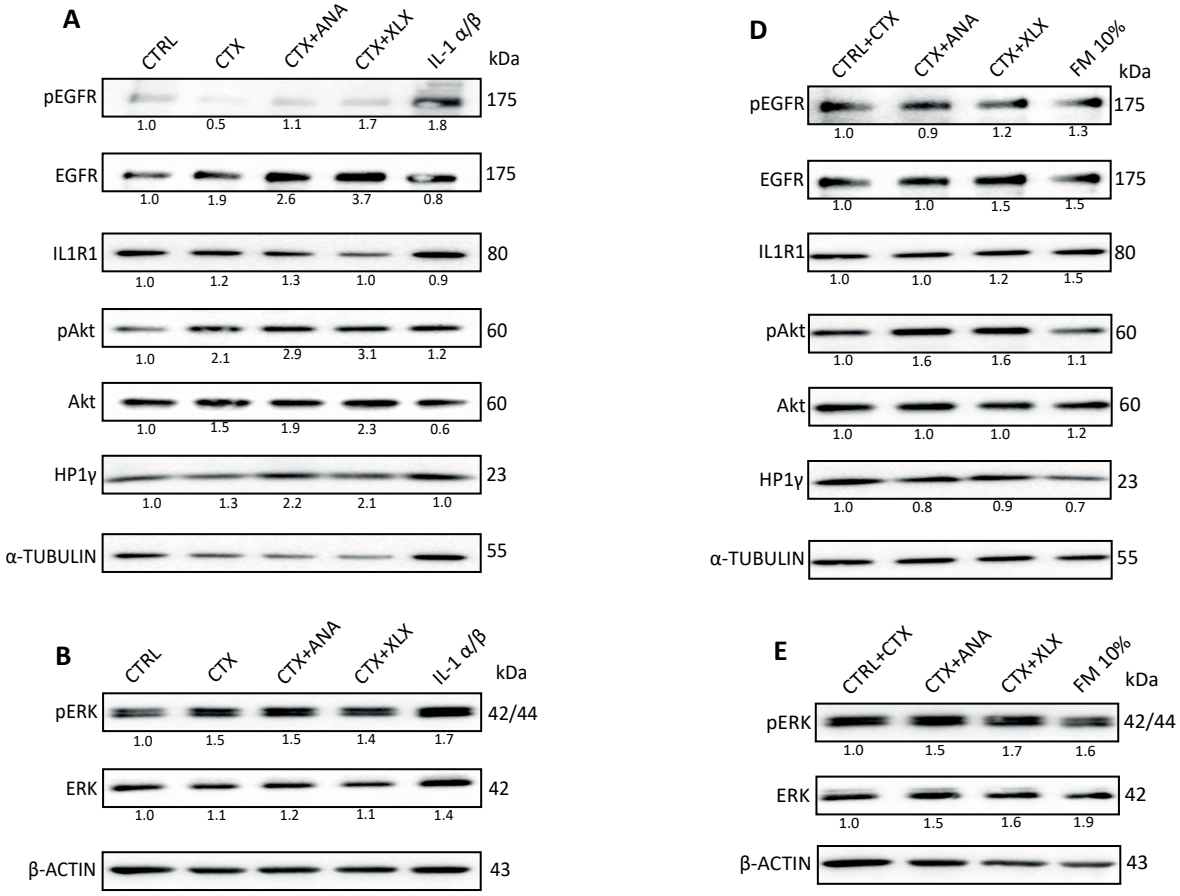
When external agents cause senescence in tumour cells, it can impede the growth of the tumour and prompt a cell cycle arrest (103). Such a form of cellular senescence can be evaluated by detecting markers associated with senescence being overexpressed. Hp1γ is one such marker, and an increase in it indicates damage to DNA (104). In fact, a family of proteins known as the Hp1 protein or heterochromatin protein, is involved in the response to DNA damage. One explanation for phosphorylated Hp1's presence in damaged cells caused by chromosomal breaks and oxidative stress is that it functions as a response signal towards the DNA repair mechanism (105). As a result, the degree of DNA damage and cellular senescence can be connected with the measurement of the Hp1 protein. After being exposed to anti-EGFR and anti-IL-1/IL1R1 inhibitors, parental and CTX-R cells were lysed, and total proteins were extracted and measured. The isolated total proteins were resolved in a nitrocellulose membrane and incubated with anti-Hp1γ (senescence marker protein). Upon quantification of bands, dark bands were observed in parental cells, and the intensity of those bands compared to control showed a two-fold increase in secretion of Hp1γ, suggesting the senescence activity in cells treated with anti-EGFR and anti-IL-1/IL1R1 compared to control cells. Such indication contemplates the combinatorial drug action that induces increased senescence marked with increased expression of Hp1γ in parental cells (figure 5A). Strikingly, no such changes with respect to expression levels of the senescence marker were observed in CTX-R cells (figure 5D) indicating the combinatorial drug action may have failed to induce genetic damage in CTX-R cells.

Interestingly, parental cells treated with anti-EGFR and anti-IL-1/IL1R1 showed an overall increase in EGFR signaling denoted by overexpressed pEGFR, pAkt, and pERK (figure 5A&B). As EGFR-mediated signaling pursues two alternative signaling cascades namely the RAS-mediated pathway and the Serine/Threonine kinase pathway, the detection of phosphorylated adaptor molecules with respect to both pathways makes it ambitious to study the precise role of anti-EGFR and anti-IL-1/IL1R1 in modulating such change. Unusually, we too detected a significant increase in pEGFR, pAkt, and pERK triggered by IL-1 α/β which seems quite strange owing to the biological role of IL-1 signaling. Such wired expression of adaptor proteins induced by treatment critical in mediating EGFR signaling makes it more curious to delineate the exact molecular mechanism behind the screen. On the other hand, a subtle increase in pEGFR, pAkt, and pERK was detected in CTX-R cells (figure 5D&E) when treated with both anti-EGFR and anti IL1R1 inhibitors.

Gene expression analysis via Real-Time PCR depicts no significant change in expression levels of IL-1 α and IL-1 β in both parental cells and CTX-R when treated with anti-EGFR and anti-IL1/IL1R1. A subtle yet not significant level of IL1R1 is observed in both parental cells and CTX-R cells when treated with CTX+XLX compared to CTX alone. This subtle increase in receptor expression again speculates the precise role of IL-1 signaling (especially IL-1 α) towards tumour homeostasis.

Upon comprehensive analysis, it becomes evident that anti-EGFR and anti-IL-1/IL1R1 indeed increase the senescence activity in both parental cells and CTX-R cells marked by an increase in the two-fold expression level of Hp1γ.

FIGURE 5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IL1R1
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pAkt
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Akt
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HP1γ
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α-TUBULIN
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B
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CTX
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CTX+XLX
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kDa
pERK
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ERK
42
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β-ACTIN
43
D
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CTX+ANA
CTX+XLX
FM 10%
kDa
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175
1.0 0.9 1.2 1.3
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IL1R1
80
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pAkt
60
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Akt
60
1.0 1.0 1.0 1.2
HP1γ
23
1.0 0.8 0.9 0.7
α-TUBULIN
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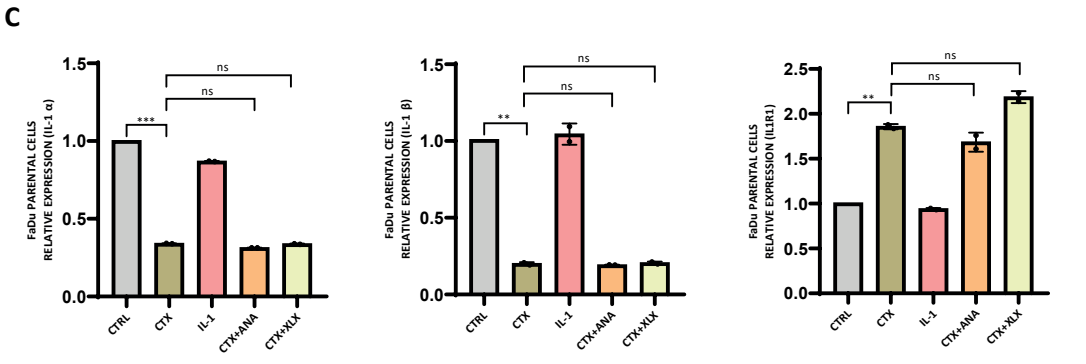
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FaDu PARENTAL CELLS RELATIVE EXPRESSION (IL-1 β)
FaDu PARENTAL CELLS RELATIVE EXPRESSION (IL1R1)
CTRL
CTX
IL-1
CTX+ANA
CTX+XLX
ns
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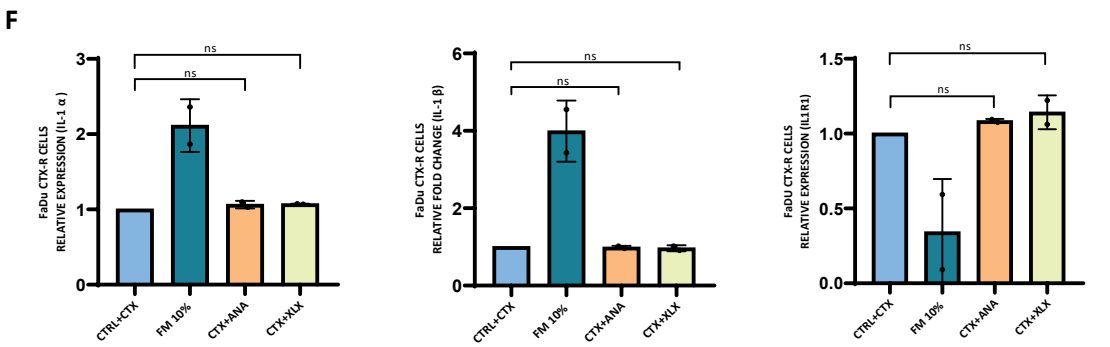
**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FaDu CTX-R CELLS RELATIVE FOLD CHANGE (IL-1 β)
FaDu CTX-R CELLS RELATIVE EXPRESSION (IL1R1)
CTRL+CTX
FM 10%
CTX+ANA
CTX+XLX
ns

FIGURE LEGENDS

Fig: 5 - IL-1 antagonists increase the relative expression of IL1R1 mRNA expression and Hp1γ.

- ***A & B –*** *Western blot analysis on FaDu parental cells revealed the induction of senescence is achieved by combinatorial drug activity marked with a two-fold increase in expression of Hp1γ (senescence marker). Interestingly, the combinatorial drug treatment of CTX (20ug/ml) + ANA (50ug/mL) and CTX (20ug/mL) + XLX (50ug/ml) showed increased phosphorylated activity of EGFR, Akt, and ERK contradicting our previous claim and the molecular mechanism behind such an adverse change is yet to be explored. Additionally, parental cells treated with IL-1 α/β (10ng/mL each) showed increased activity of pEGFR, pAkt, and pERK yet again increasing our interest.* α *Tubulin and* β *Actin were served as internal control genes.*

- ***C*** *– Relative mRNA expression analyses of IL-1* α*, IL-1* β *and IL1R1 from parental cells showed the combinatorial drug activity appeared to have no overall significant difference in upregulating or down-regulation of the expression levels of IL-1 ligands and receptor while a subtle increase in the relative expression of IL1R1 was observed when the parental cells are treated with CTX (20ug/mL) + XLX (50ug/mL). The B2M gene served as internal control.*

- ***D & E*** *– Western blot analysis on FaDu CTX-R cells showed no significant change with respect to the expression levels of senescence marker protein Hp1γ. A significant increase in the activity of pAkt and pERK was observed in cells treated with CTX (20ug/mL) + ANA (50ug/mL) and CTX (20ug/mL) + XLX (50ug/mL).* α *Tubulin and* β *Actin served as internal controls.*

- ***F –*** *Relative mRNA expression analyses of IL-1* α*, IL-1* β*, and IL1R1 from CTX-R cells showed the combinatorial drug activity appeared to have no overall significant difference in up-regulating or down-regulation of the expression levels of IL-1 ligands and receptor while a subtle increase in the relative expression of IL1R1 was observed when the CTX-R cells treated with CTX (20ug/mL) + XLX (50ug/mL). The B2M gene served as internal control.*

5.6 Simultaneous neutralization of EGFR and IL-1 accelerates decrease in the overall volume of spheroids

The three-dimensional Multicellular Tumour Spheroid Model (MCTS) is a quasi-in vivo research model system that has been extensively employed in cancer research to examine different neoplasm behavioural patterns (106). The cells are grown in an anchorage-independent ambient achieved either by coating the exterior surface of the wells with anti-adhesion agents like agarose or directly employing adhesion-free culture plates inhibiting the cell adhesion. Such a 3-D environment mimics the growth of spheroids as in an in vivo tumour. The previously mentioned spheroid model makes it easier to comprehend the kinetics of drug activity and the modality of drug penetration in a three-dimensional environment, thus facilitating to figure out differences in drug activity between in vitro and in vivo treatment (107). The MCTS model further serves as a transitional model to predict the therapeutic potential of medications in practical terms of prediction before actual in vivo models are employed (108).

Under 3D growing conditions, the effect of combinatorial drug potential in reducing the overall size of spheroids in terms of volume was evaluated in both parental cells and CTX-R cells. In wells coated with 2% agarose beforehand, parental cells and CTX-R cells were seeded. These agarose-coated wells stopped the tumour cells from forming monolayers and adhering to the bottom of the wells. Appropriate treatment is added as per the setup along with medium and the incubated. Upon incubation, the cells started to aggregate with one another as a single unit, ultimately forming one single compact spheroid. The size of the formed spheroids was meticulously examined. Subsequently, after a 12-day incubation period, each spheroid is photographed at a 4X magnification, and the total volume of the spheroids is measured to determine the relative spheroid volume for each spheroid.

When treated with anti-EGFR and anti-IL-1/IL1R1 combined, spheroids derived from parental cells exhibited a milder response to treatment (figure 6A). Additionally, the number of dead cells floating around the spheroids treated with anti-EGFR and anti-IL-1/IL1R1 was significantly higher than the number of dead cells derived from the control cells, suggesting that the drugs activity is highly effective in cells present in the spheroids peripheral layer. When treated with anti-EGFR and anti-IL1/IL1R1, the total volume of spheroids derived from CTX-R cells did not significantly decrease (figure 6B).

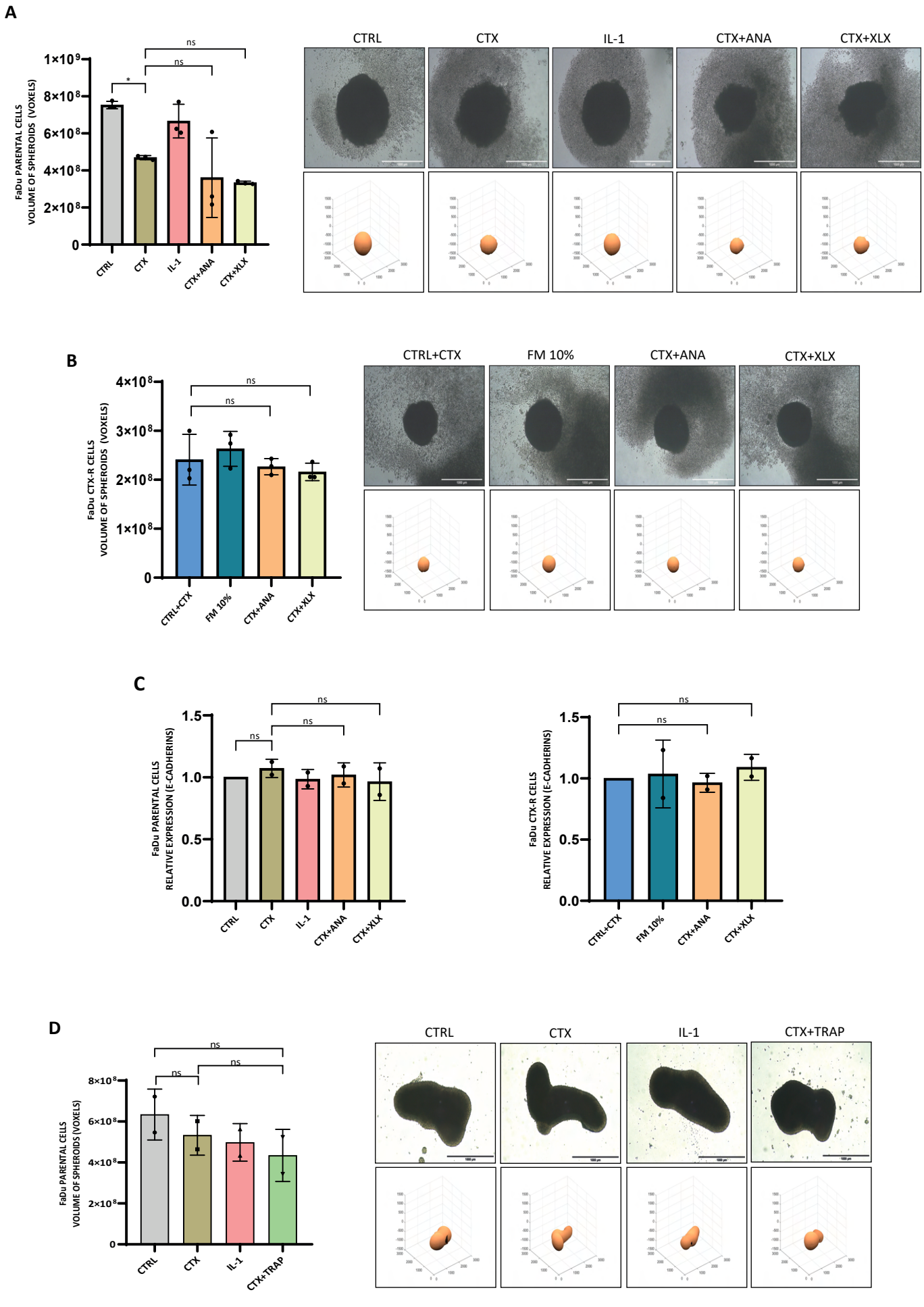
Surprisingly, it was discovered that the spheroids' overall integrity had not been derailed, indicating that the medications are unable to suppress the expression of E-cadherins. We examined the relative expression levels of E-cadherins in CTX-R cells and parental cells to substantiate our claim (figure 6C), and we discovered no noticeable difference in E-cadherin expression levels.

- **h-TRAP IL-1 in combination with Cetuximab proved to be effective in shrinking the volume of spheroids in a brief treatment period.**

The combined use of cetuximab and h-TRAP IL-1, a laboratory-developed anti-IL-1 α/β antagonist (decoy receptor), resulted in a more rapid and effective reduction in the total volume of spheroids produced from parental cells. Parental cells were treated with CTX and h-TRAP IL-1 after being seeded in agarose-coated wells. Images of the spheroids were taken at 4X magnification following a 96-hour treatment period. After examination, it was found that TRAP in combination with CTX performed more efficiently than CTX alone in decreasing the total volume of spheroids (figure 6D). This considerable yet non-significant reduction in spheroidal volume was clearly visible and provided additional evidence of TRAP's role as a critical modulator in augmenting the overall efficacy of traditional anti-EGFR inhibitors.

On a holistic approach, it is convincing that combinatorial drug activity targeting both EGFR and IL-1/IL1R1 simultaneously proved to be quite effective with respect to shrinking the tumor volume if employed in vivo. On the contrary, the combined activity of drugs deplorably failed to disintegrate or disrupt the overall integrity of spheroids.

FIGURE 6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6×10⁸
4×10⁸
2×10⁸
0
ns
ns
*
CTRL
CTX
IL-1
CTX+ANA
CTX+XLX
CTRL
CTX
IL-1
CTX+ANA
CTX+XLX
B
FaDu CTX-R CELLS VOLUME OF SPHEROIDS (VOXELS)
4×10⁸
3×10⁸
2×10⁸
1×10⁸
0
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ns
CTRL+CTX
FM 10%
CTX+ANA
CTX+XLX
CTRL+CTX
FM 10%
CTX+ANA
CTX+XLX
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0.0
ns
ns
ns
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IL-1
CTX+ANA
CTX+XLX
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1.0
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FM 10%
CTX+ANA
CTX+XLX
D
FaDu PARENTAL CELLS VOLUME OF SPHEROIDS (VOXELS)
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6×10⁸
4×10⁸
2×10⁸
0
ns
ns
ns
CTRL
CTX
IL-1
CTX+TRAP
CTRL
CTX
IL-1
CTX+TRAP

FIGURE LEGEND

Fig: 6 - Additive action of IL-1/IL1R1 and CTX shrinks the overall volume of spheroids in parental cells.

The Spheroids are incubated for 12 days with treatment, pictured at 4 X magnification, and processed in ImageJ. Processed images were analyzed for their volume by ReVisP (a software specifically designed to calculate 3D spheroid volume from 2D spheroid images). The calculated volume of spheroids is thus exhibited in units of voxels and their statistical significance is determined using GraphPad Prism.

A. *Spheroids derived from parental cells displayed an overall reduction in their volume upon being treated with CTX (20ug/mL) + ANA (50ug/mL) and CTX (20ug/mL) + XLX (50ug/mL) compared to CTX (20ug/mL) alone. Statistical significance * $p < 0.05$.*

B. *CTX-R spheroids showed no significant yet subtle decrease in the overall volume of spheroids.*

C. *Relative mRNA expression analysis of E-cadherins. from both parental cells and CTX-R describes no difference in relative expression levels of E-cadherins influenced by various treatment modules in both parental cells and CTX-R cells, thus explaining the continued maintenance of the overall integrity of spheroid around the globe.*

D. *h TRAP IL-1 showed a subtle shrinkage in the overall volume of spheroids. h-TRAP (20ug/mL) in combination with CTX (20ug/mL) showed an additive effect by shrinking the total volume of spheroids compared to CTX (20ug/mL) alone.*

5.7 Anti-EGFR and anti-IL-1/IL1R1 induce credible loss of overall cellular identity

Treatment-induced cellular plasticity serves as a benchmark for assessing the impact of medications that can alter a cell's phenotype (109). One of the malignant characteristics of tumour cells is their ability to dedifferentiate; such drug-induced dedifferentiation can have beneficial or adverse consequences (110). Defective differentiation induced by drug activity may be exploited in cancer treatment (111) and, such an approach was attempted to gain knowledge on the role of anti-EGFR and anti-IL-1/IL1R1 drugs in persuading cancer cells to undergo morphological changes.

Loss of cellular integrity is quite evident in both parental cells and CTX-R cells treated with both anti-EGFR and anti-IL-1/IL1R1 inhibitors. The disruption in overall changes in cellular identity in terms of disorientated cellular morphology primarily helps to ascertain the degree of drug penetration deep inside the spheroids and helps to further ascertain the modus of drug action in a 3-D environment. Both parental cells and CTX-R cells were seeded in pre-coated autoclaved agarose wells and treated with anti-EGFR and anti-IL-1/IL-1R1 along with IL-1 α/β for a period of 12 days. After 48 hours of incubation, the cells started to well aggregate and formed distinct spheroids marked with well-confined boundaries. After the treatment period, the spheroids were collected, fixed with 10% formalin, embedded in parafilm wax, sectioned, and subjected to simple Immunohistochemistry staining with Eosin and Haematoxylin. Upon analysis, the spheroids derived from parental cells treated with both anti-EGFR and anti-IL-1/IL1R1 appeared to show rigid circular cell morphology compared to control cells (figure 7A). Such rigid cellular morphology is induced due to the cumulative action of drugs, indicating the formation of apoptotic bodies or inducing apoptosis-mediated cell death. Surprisingly, the spheroids treated with IL-1 α/β showed morphologically distinct healthy cells with well-demarcated cellular morphology and well-established boundaries, thus showing the overall spheroid integrity was well maintained indicating IL-1 ligand-mediated signaling plays a crucial role in exaggerating tumorigenesis in Head and Neck cancer.

On the other hand, total loss of cellular identity is observed in CTX-R cells treated with both anti-EGFR and anti-IL-1/IL1R1 (figure 7B). Although the role of treatment-induced morphological changes is not well defined in our case, it can be attributed in two ways. On a positive scale, it can be traced back to the action of drugs inducing necrosis-mediated cell death while on a negative side, it can be annotated that the action of drugs induced the transition of tumor

cells further into an aggressive phase marked with lack of cellular identities. If the latter case serves to be true, we could conclude the combinatorial drug action may further increase the severity of treatment and the combined treatment modality can be restricted only to first-line treatment strategy.

FIGURE 7

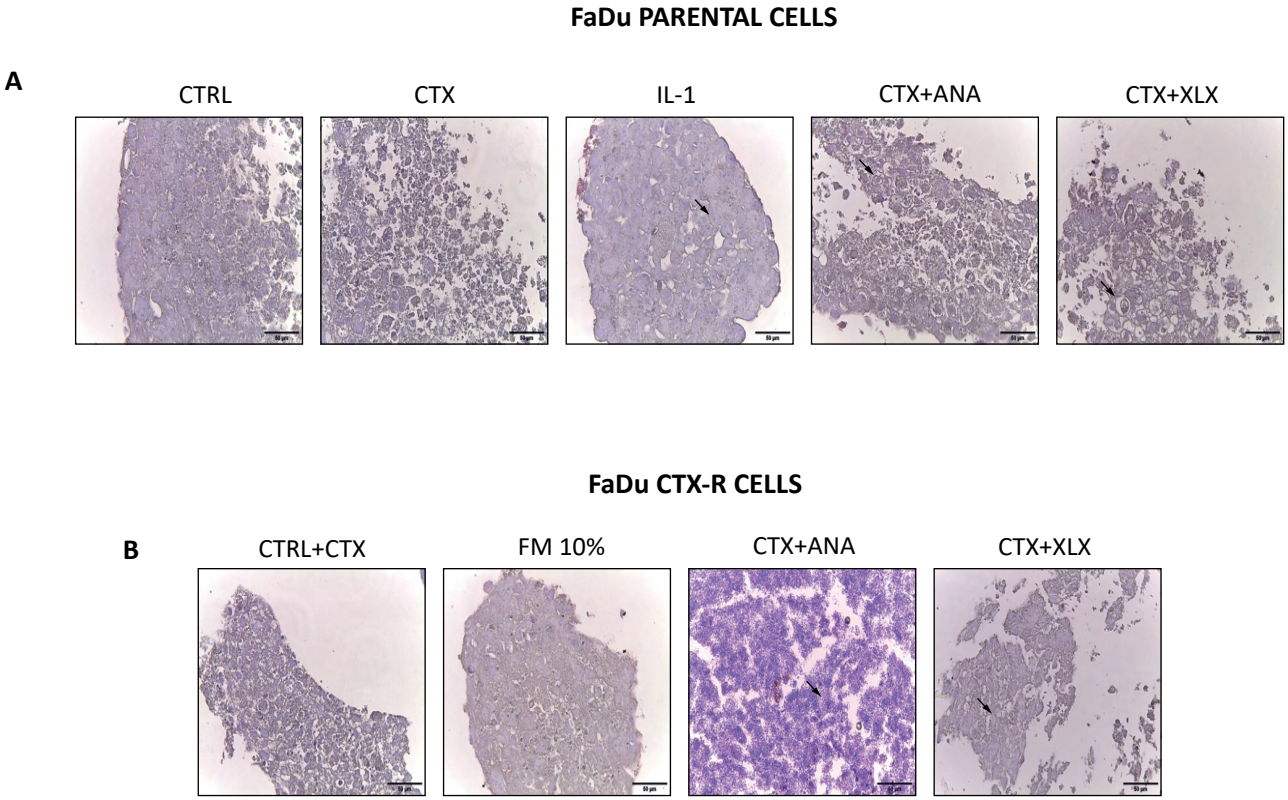
FaDu PARENTAL CELLS
A
CTRL
CTX
IL-1
CTX+ANA
CTX+XLX
FaDu CTX-R CELLS
B
CTRL+CTX
FM 10%
CTX+ANA
CTX+XLX

FIGURE LEGEND

Fig: 7 - Loss of cellular integrity is quite evident in both parental cells and CTX-R cells treated with both anti-EGFR and anti-IL-1/IL1R1 inhibitors.

Both spheroids derived from parental cells and CTX-R were treated with the appropriate treatment module for a period of 12 days, fixed with 10% formalin followed by embedding of spheroids in parafilm. Fine sections were made through microtome followed by Eosin and Haematoxylin staining.

- ***A.*** *Upon staining, Parental cells displayed a compelling change in the loss of cellular identity marked with cyst-like hardened cell boundaries when treated with CTX (20ug/mL) + ANA (50ug/mL) and CTX (20ug/mL) + XLX (50ug/mL). Cells treated with CTX (20ug/mL) alone showed an increase in cellular death with distorted cellular morphology. Parental cells treated with IL-1 α/β (10ng/mL each) showed enhanced cellular integrity compared to control cells.*

- ***B.*** *Total loss of cellular identity is quite evident in CTX-R cells treated with CTX (20ug/mL) and XLX (50ug/mL).*

5.8 Anti-IL-1/IL1R1 constitutes changes in the mechanobiological aspects of tumour cells

The two traditional characteristics that a tumour cell has to possess in order for the disease to successfully spread are invasiveness and metastasis. Such characteristics ought to depend on mechanobiological characteristics in addition to the genetic and molecular signatures of a tumour cell. Analysing the function of mechanobiological parameters as potential biomarkers to predict overall disease progression has been a major focus in recent studies. It is believed that a tumour cell mechanobiological or biomechanical characteristics have a profound effect on how successfully it invades surrounding organs and/or spreads to other locations (112,113). Using cutting-edge technologies or Artificial Intelligence (A.I.) based methodologies empowers the understanding of these mechanobiological properties and ensures that drugs can induce either beneficial or detrimental paradigms, thereby altering the overall mechanobiological properties of cancer cells (114). Solid neoplasms can also be defined by the proactive role of biomechanical properties to tumour progression; and the presence of a treatment regimen may modify these implications (115). The two main components of our study that we focused are the morphology and motility characteristics of cells. The total potential for invasion and metastasis of the neoplasm is determined by both of these factors, thus, measuring the variation in these features triggered by combinatorial drug action can be of serious consideration when developing future treatment strategies. In this work, we considered changes brought about by the treatment in terms of morphological parameters like cell thickness and cellular sphericity, as well as motility parameters like instantaneous velocity, track speed, and confinement ratio.

Following the administration of the appropriate treatment modules, each morphological parameter was examined in both parental cells and CTX-R cells. When parental cells were treated with anti-EGFR and anti-IL-1/IL1R1 simultaneously, there was a significant decrease in both cellular thickness and cellular sphericity (figure 8A). The reduction in cellular sphericity and thickness is due to general cell shrinkage, which is a prelude to necrosis-mediated cell death and negatively affects the invasive and metastatic characteristics of cancer cells. However, CTX-R cells do not exhibit any appreciable alterations in cellular thickness or sphericity (figure 8B), suggesting that the drugs may not have been able to alter the mechanobiological characteristics of CTX-R cells. Upon analysing the motility parameters that are deemed to be crucial for upholding metastatic properties, we measured instantaneous velocity, track speed, and confinement ratio in both parental cells and CTX-R cells. When treated with both anti-EGFR and anti-IL-1/IL1R1 compared to anti-EGFR alone, both parental cells and CTX-R cells displayed a statistically significant decrease in track speed and instantaneous

velocity (figure 8 C&D); however, with respect to the confinement ratio parameter, neither parental cells nor CTX-R cells showed any significant changes.

With the above facts, it is premature to determine with precision whether treatment-induced effects are associated with a favourable or unfavourable prognosis, despite our thorough analysis showing that combinatorial drug activity of both anti-EGFR and anti-IL-1/IL1R1 influenced the overall biomechanistic properties of both parental cells and CTX-R cells. More research into these modifications can clarify their exact behavioural patterns and the long-term effects they will have on prognosis related to treatment.

FIGURE 8

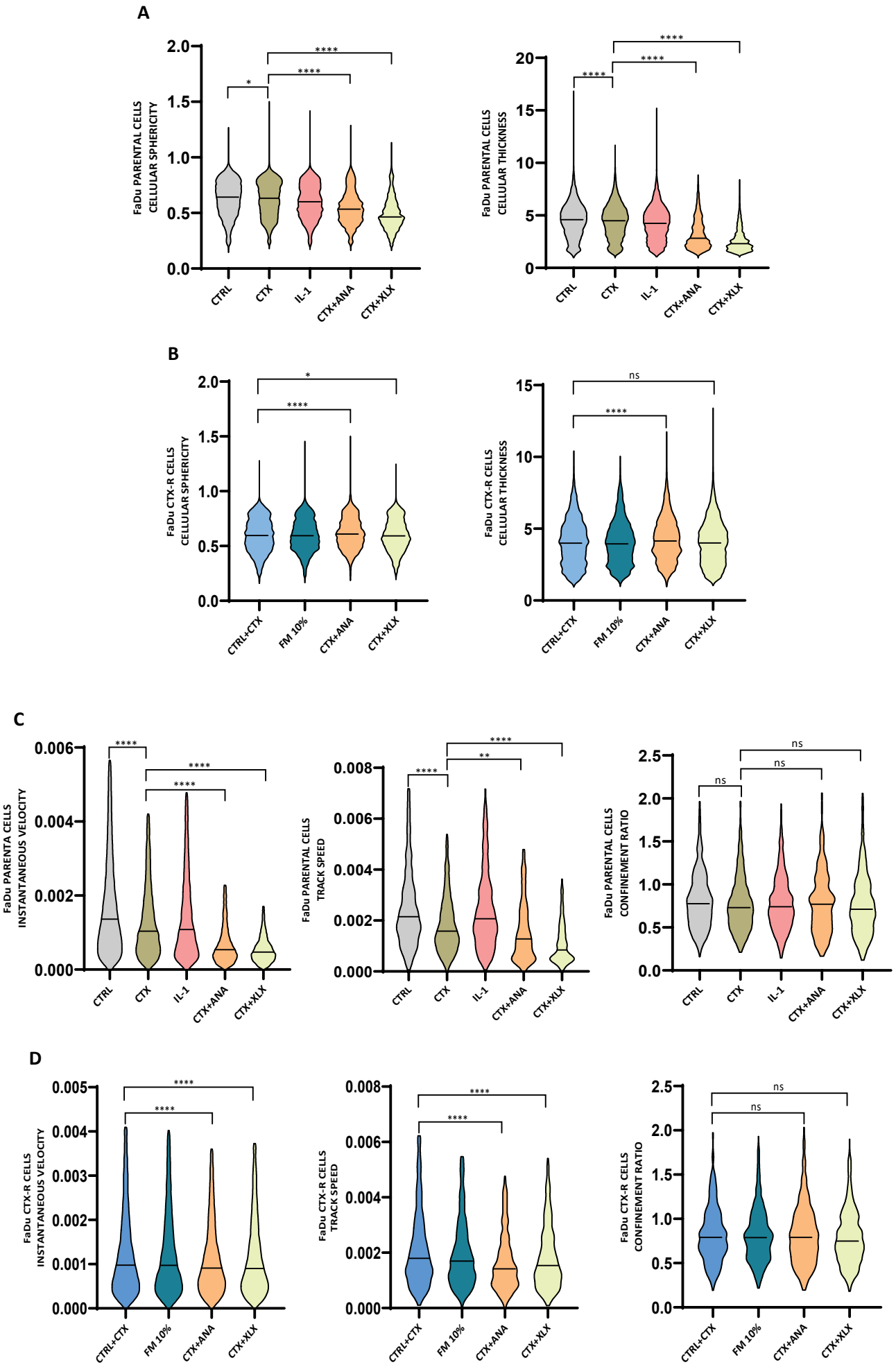
A
FaDu PARENTAL CELLS CELLULAR SPHERICITY
FaDu PARENTAL CELLS CELLULAR THICKNESS
CTRL
CTX
IL-1
CTX+ANA
CTX+XLX
B
FaDu CTX-R CELLS CELLULAR SPHERICITY
FaDu CTX-R CELLS CELLULAR THICKNESS
CTRL+CTX
FM 10%
CTX+ANA
CTX+XLX
C
FaDu PARENTA CELLS INSTANTANEOUS VELOCITY
FaDu PARENTAL CELLS TRACK SPEED
FaDu PARENTAL CELLS CONFINEMENT RATIO
D
FaDu CTX-R CELLS INSTANTANEOUS VELOCITY
FaDu CTX-R CELLS TRACK SPEED
FaDu CTX-R CELLS CONFINEMENT RATIO

FIGURE LEGENDS

Fig: 8. IL-1/IL1R1 blockade alongside anti-EGFR inhibitor induces detrimental changes in mechanobiological properties in parental cells.

*Biomechanical properties with respect to cellular morphology parameters and cellular motility parameters were assessed through Livecyte Real-Time analysis. Both parental cells and CTX-R cells were seeded in a 96-well plate as per the experimental design and incubated overnight. The following day, appropriate treatment was added and incubated for a 48-hour period and incubated inside the Livecyte analyzer. Pictures were taken at regular intervals of 30 minutes and calculations were made based on the software algorithms for each aforementioned properties. Statistical significance * P < 0.05; ** P < 0.01; **** P < 0.0001*

- ***A & B*** *– Morphological properties in terms of cell thickness and cellular sphericity were analyzed in both parental cells and CTX-R cells. Both parental cells and CTX-R cells showed a statistically significant decrease in morphological properties induced by combinatorial drug treatment of CTX (20ug/mL) + ANA (50ug/mL) and CTX (20ug/mL) + XLX (50ug/mL) compared to CTX (20/ug/mL) alone.*

- ***C & D*** *– Motility properties in terms of instantaneous velocity, track speed, and confinement ratio were assessed in both parental cells and CTX-R cells. Again, both parental cells and CTX-R cells showed an increased sensitivity towards combinatorial drug treatment of CTX (20ug/mL) + ANA (50ug/mL) and CTX (20ug/mL) + XLX (50ug/mL) specifically marked in instantaneous velocity and track speed properties. No such significant changes were observed in both parental cells and CTX-R cells with respect to properties concerning confinement ratio.*

6. DISCUSSION

Tumorigenesis involves an armamentarium of factors facilitating the cross-talk among several cellular types. This in association with other genetic events, namely mutations of onco-suppressor genes and activation of driver oncogenes, ultimately leads to tumour development. Moreover, such factors trigger a wave of signals devoid under normal circumstances overhauling the entire landscape of tumorigenesis and leading to the failure of the most conventional therapies. Pro-inflammatory cytokines may play a crucial role in the early stage of tumorigenesis and many research studies have depicted the contentious role of pro-inflammatory cytokines in chronic inflammation and their tie-up towards cancer progression (93). A relevant and well-known pro-inflammatory cytokine that gained traction is IL-1 and its related signaling cascades in tumorigenesis (94).

Over the two years of my PhD study, we aimed to delineate and establish the role played by IL-1 in exaggerating the overall tumour burden in Head and Neck Cancer patients and their role in impairing the efficacy of conventional therapies. Head and Neck Squamous Cell Carcinoma is marked by overexpression of EGFR, making it a prime target for treatment. Cetuximab – a chimeric monoclonal antibody (FDA-approved) binds to the extracellular domain of the EGF receptor and impedes the binding of the EGF ligand and its respective downstream signaling cascade (116), yet, the development of treatment-induced resistance in patients is a worrisome factor. To circumvent, simultaneous targets on both IL-1 and EGFR may increase treatment efficacy and increase the Overall Survival and/or Progression Free Survival in patients. Past studies have shown the simultaneous employment of both anti-EGFR and anti-IL-1 drugs, might mitigate agony and increase positive response in patients. Of note, inhibiting or curtailing IL-1 alongside EGFR through systemic introduction possibly triggers severe autoimmune consequences in patients as IL-1 signaling holds crucial sway in immune modulation. Here, starting from *in vitro* cell line models, we measured the effects of combinatorial drug action targeting both EGFR and IL-1/IL1R1 axis, by recording the induced changes.

Thus, we first determined to investigate the therapeutic potential of both anti-EGFR and anti-IL-1 inhibitors employed simultaneously by measuring the influence of the drug combination in hindering cellular proliferation. Upon analysis, we identified the combinatorial drug action indeed, decreased overall cell proliferation in both parental cells and CTX-R cells marked by a decrease in the number of cellular colonies formed with CTX treatment alone and CTX in combination with anti-

IL-1 treatments. Secondly, we determined to understand the effect of drugs with respect to induction of senescence-mediated cell growth arrest. Again, parental cells showed a heightened response towards combinatorial drug action with a higher percentage of senescent positive cells compared to a number of senescent cells induced by CTX alone. Consistently, no senescence activity was detected in CTX-R cells, probably due to limited exposure of CTX-R cells to drug concentration and/or short treatment duration.

The next phase of our investigation focused on analysing the molecular signaling and the implications induced by the combinatorial treatment strategy. Gene expression analyses by Real-Time PCR revealed a decrease in IL-1 α/β ligands influenced by treatment in both parental cells and CTX-R cells, when treated with both anti-EGFR and anti-IL-1 (IL-1 α inhibitor), thus suggesting the role of IL-1 signaling as parallel pathway when canonical EGFR signaling is compromised. Subsequently, we aimed to decipher any treatment related alterations made to canonical molecular signaling cascades by measuring the expression load of proteins. Upon analyses by western blot, we found a two-fold increase in Hp1γ protein (a senescence marker) (104) confirming that the combinatorial treatment is indeed capable of inducing senescence specifically in parental cells. As noted, no such change in expression levels of Hp1γ was found in CTX-R cells in line with our previous investigations. On the contrary, we found that IL-1 α/β ligands were found to enhance EGFR-mediated signaling marked with an increase in pEGFR, pAkt, and pERK in parental cells induced by combinatorial drug treatment. Such an upregulated expression of receptor phosphorylation and adaptor molecules suggests the presence of a direct cross-talk between EGFR and IL-1 signaling axes, thus further complicating the efforts to pinpoint the molecular mechanisms behind the drug actions.

As the main aim of our investigation is to determine the combinatorial drug efficacy in patients, we determined to measure the efficacy of drug action in a 3D environment. Thus, we employed a 3D Multicellular Tumour Spheroid Model (106) by culturing *in vitro* FaDu cells, eventually leading to the formation of spheroids. Such spheroids derived from parental cells when treated with the combination of drugs showed a subtle decrease in overall spheroid volume further confirming the results obtained in 2D settings. On the other hand, CTX-R derived spheroids failed to respond to the treatment in 3D setting, which again goes in line with our previous findings.

Tumour invasion and metastasis are part and parcel properties of tumour cells that facilitate the rapid spread of disease across the axis. (112, 113). Thus, we measured the changes in

morphological and motility parameters in time induced by drug treatment in both parental cells and CTX-R cells. Morphological parameters like cellular thickness, cellular sphericity, and motility parameters like track speed, confinement ratio, and instantaneous velocity were found to be greatly impaired due to combinatorial drug activity explicitly in parental cells. Mixed responses concerning analyses of the above-mentioned parameters were recorded in CTX-R cells.

Fletcher and his colleagues have demonstrated that Erlotinib, a Tyrosine Kinase inhibitor (TKi) accelerates the expression of pro-inflammatory cytokines mediated by oxidative stress-related NOX4. Such signaling prudence often poses a bottleneck in treating HNSCC exhibiting hyperexpression of EGFR thereby watering down the efficacy of EGFR inhibitors (117). Thus, to further ascertain the previous claim, *Koch et al.,* deciphered that Erlotinib indeed has the capacity to induce IL-6 (pro-inflammatory cytokine) secretion with a noted reduction of drug efficacy in HNSCC cell lines. Additionally, in vitro by deleting MyD88 (an adaptor protein molecule involved in down-stream Toll-Like Receptor-mediated pathways), expression of Erlotinib-mediated secretion of IL-6 is curtailed, thus leading to the increase in anti-tumour efficacy of Erlotinib, tested also in animal models, (*in vivo)*. Besides, an uptick in the expression of IL-1 α mRNA mediated by Erlotinib is noted in HNSCC cell lines, aiding tumour progression in HNSCC. In line, blocking IL-1α and IL1R1 improves the efficacy of both cetuximab and Erlotinib (118). But the field remains still controversial, indeed *Espinosa-Cotton et al.,* claimed that IL-1 α expression may trigger anti-tumour efficacy of EGFR inhibitor namely cetuximab *in vivo* and a total blockade of IL-1 α showed little to no effect in decreasing the anti-tumour activity of cetuximab (119).

In summary, our findings suggest that *in vitro*, the combinatorial activity of both anti-EGFR and anti-IL-1/IL1R1 inhibitors simultaneously enhances the overall efficacy of the treatment, by the overall deregulation of tumour growth. Additionally, the combinatorial drug treatment aids in decreasing the factors responsible for invasiveness and metastasis, such as cellular thickness, cellular sphericity, and motility like track speed, confinement ratio, and instantaneous velocity. These findings are found to be promising in parental cells, while CTX-R cells showed a mixed response towards the combinatorial treatment strategy. These controversial results could suggest that the acquisition of the resistant phenotype involves the activation of a bypass pathway, which is independent of the EGFR/IL1 axis neutralization. A limiting factor of this study is the employment of a single cell line rather than multiple cell line models, although these findings were obtained in 2D and 3D settings. Moreover, for a better comparison and a parallel assessment of these findings it is mandatory to test also *in vivo* models (which may be included in future study).

7. CONCLUSION

Summing up, we hypothesize that a molecular cross-talk between IL-1 and EGFR exists, knotted towards the development of resistance to monoclonal antibody, namely cetuximab in Head and Neck Cancer. Conventional treatment modalities often fail at a later stage marked by the over-expression of IL-1 ligands and IL1R1, which represent a negative prognostic factor and often correlate with poor survival. Therefore, we suggest that simultaneous targets of both EGFR and IL-1/IL1R1 should be the prime focus of the treatment strategy. Combinatorial drug action, as previously discussed impedes the development of tumor progression, yet, such an approach is far from reality owing to various limiting factors.

As IL-1 signaling is correlated to the lack of response to the traditional treatment modalities, complete blockade may cause patients to experience unintended side effects. It is important to never underestimate the pleiotropic nature of IL-1's function in regulating the primary immune response. The systemic approach of introducing anti-EGFR and anti-IL-1/IL1R1 may dilute the overall effect of the drugs by acting on other non-players thus bringing down the overall needed response. To circumvent this, a targeted approach could be considered to increase the treatment efficacy and this can be achieved by delivering anti-EGFR and anti-IL1/IL1R1 directly at the site of the tumour. Such an approach may concentrate the drug activity to many folds and increase the betterment in patients.

Citing from Roberts Frost's *''And miles to go before I Sleep, And miles to go before I Sleep''*, there are various parameters that need to be independently verified before actually employing the combinatorial drug treatment strategy in patients. It is imperative that cancer is a disease with multifactorial hands-on deck, it is nearly impossible to target and destroy each factor responsible for overall tumour endurance. All we can focus on is increasing the efficacy of the treatment by modulating/amending present treatment strategies with the prime focus on increasing the qualitative Overall Survival and extended Progression-Free Survival with less to no negative impact on patient well-being.

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