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TESI DI DOTTORATO

"Neuropilin-1 in head and neck cancer"

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Abstract

Head and neck squamous cell carcinoma (HNSCC), the sixth most common tumor worldwide, accounts for more than 550,000 new cases and 380,000 deaths per year. Patients diagnosed with locally advanced disease often require multimodal treatment approaches. Chemoradiation, with high cisplatin dose, remains the standard of care for this category of patients. Molecular target therapies have been introduced, such as the combination of the Epidermal Growth Factor Receptor (EGFR) monoclonal antibody (cetuximab) with cisplatin and 5-fluorouracil for the treatment of recurrent or metastatic HNSCC. EGFR is one of the most studied molecular target, since it is overexpressed in about 90% of HNSCC and Neuropilin-1 (Nrp-1), that is a co-receptor of EGFR, represents an interesting candidate to investigate in HNSCC.

Nrp-1 has been first discovered as regulator of the nervous system development, acting together with Plexins as co-receptor for Semaphorins (SEMAs); then, Nrp-1 has been identified as receptor for several Vascular Endothelial Growth Factors (VEGFs). Recent findings determined that Nrp-1 is able to enhance the signaling activated by the ligands and tyrosine kinase receptors (RTKs) interaction. In particular, upon ligand stimulation, Nrp-1 can elicit EGFR clustering, endocytosis and signaling. Consequently, Nrp-1 results involved in multiple oncogenic processes, such as cellular proliferation, survival, invasion and migration. Few evidences reported a Nrp-1 contribution in response to cancer therapy, even if its role in chemotherapy still is in need of further investigation.

In this study, we aim to address three main issues: 1) to investigate in a study population of human head and neck cancers (N=217), the Nrp-1 expression and its correlation with the clinicopathological features of patients, by statistical analysis; 2) to explore, *in vitro*, in stable Nrp-1 silenced head and neck cancer cells, the Nrp-1 contribution to cisplatin sensitivity; 3) to analyse the Nrp-1 role in sustaining the cisplatin-induced EGFR activation.

In the HNSCC cohort of surgical samples, organized in tissue microarrays, we investigated the Nrp-1 expression by IHC analysis, showing the Nrp-1 overexpression in malignant tissues compared to the normal counterpart. Performing survival and multivariate analysis, Nrp-1 expression resulted an independent prognostic factor for HNSCC patients. By transduction with lentiviral vectors, we obtained the Nrp-1 depleted HNSCC cellular models. Interestingly, we observed the increase of cellular sensitivity to cisplatin treatment in the Nrp-1 silenced cells, compared to control cells, transduced with the empty lentiviral

vector. Finally, we showed that Nrp-1 is able to sustain the cisplatin induced EGFR activation together with the tyrosine-protein kinase Src, a mechanism proposed to serve as a cell-survival response to cytotoxic stress.

In conclusion, these results propose Nrp-1 as a novel prognostic marker for HNSCC. Furthermore, we provide preliminary evidences of Nrp-1 contribution to cisplatin sensitivity. Additionally, we showed, for the first time, the Nrp-1 ability to enhance the cisplatin induced EGFR activation, expanding the repertoire of signalling processes involving Nrp-1 and suggesting the need of further investigations on Nrp-1 as a suitable target for HNSCC therapies.

CHAPTER I

-INTRODUCTION-

1. Introduction

1.1 Head and neck cancer

1.1.1 Epidemiology

Head and neck cancer is the sixth most common malignancy in the world and the 95% of these cancers are squamous cell carcinomas (1). HNSCC arise from the mucosal epithelium of the upper airway and food passages (oral cavity, oropharynx, larynx and hypopharynx) (Figure 1). The approximate distribution of head and neck cancer by anatomic sites is of 25 % for pharynx, 31 % for larynx and 44 % for oral cavity (Figure 2). Oral cavity includes the lips, alveolar ridge, floor of mouth, oral tongue, hard palate, retromolar trigone and buccal mucosa. Cancer of the oral cavity and lip usually occurs in males after the fifth decade of life (2) (3). The mean age is in the fifth and early sixth decades in Asiatic populations, compared to the seventh and eighth decades in North American populations (4). On the other hand, oropharyngeal cancers occur at ages under the fourth decade of life (4) (5). This trend appears to continue in the next years, due to the increase of high-risk HPV infections, which now cause approximately the 70% of all oropharyngeal cancers in the USA (6) (7).

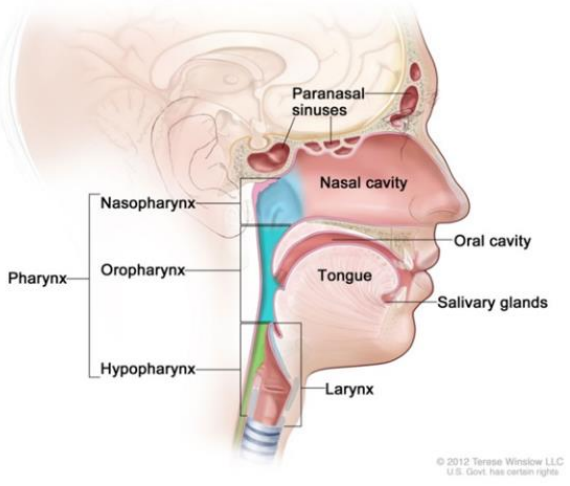


Figure 1. Schematic representation of head and neck anatomic sites.

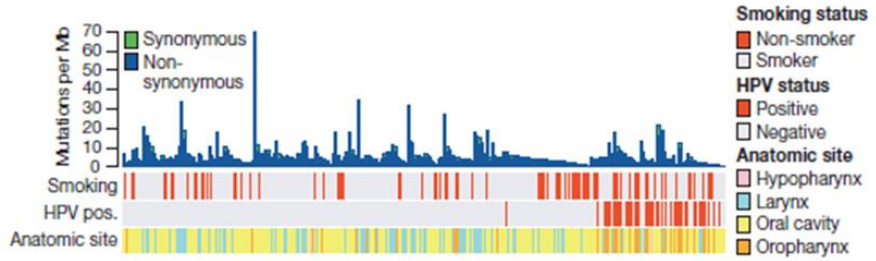


Figure 2. Schematic representation of head and neck anatomic sites distribution in tumors. The Cancer Genome Atlas Research Network, 2015.

The yearly worldwide incidence is of approximately 640,000 new cases for male and of 207,000 new cases for female (8). Estimated age-standardised incidence/mortality rates for oral cavity and lip cancers (7.0/2.3 and 2.6/0.6 per 100,000 per annum, among males and females, respectively) are higher in more developed regions compared to those in less developed regions (5.0/2.8 and 2.5/1.4 per 100,000 per annum). Likewise, the estimated rates for cancers of oropharynx and tonsils among males (7.5/2.5 per 100,000 per annum) and females (2.7/0.5 per 100,000 per annum) resulted the highest in Western Europe. Despite great advances in surgery, radiotherapy, chemotherapy and recently immunotherapy, the 5-year survival rate for HNSCC is still poor, with 40–50% mortality (9). According to 'Globocan' the estimated number of deaths reported was 97,919 among males and 47,409 among females and about the 66% of cancer mortality are reported in Asia, followed by Europe (18.4%), Africa (6.1%), Latin America and the Caribbean (5.1%) (Figure 3) (8) .

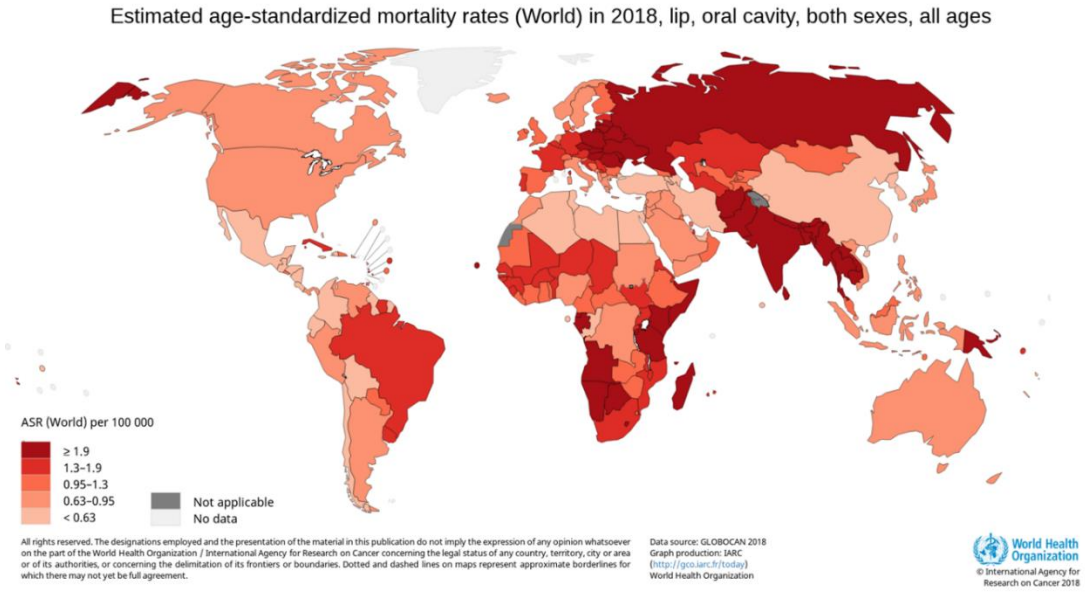


Figure 3. Head and neck cancer mortality. Age-standardized ratios. http://globocan.iarc.fr

1.1.2 Risk factors

The risk factors for HNSCC are tobacco consumption (smoked and 'smokeless'), the chewing of areca nut and heavy use of alcoholic beverage. Currently, sustained infection with 'high-risk' genotypes of the human papillomaviruses (HPVs) (particularly HPV-16 and HPV-18) causes a substantial and rising percentage of these tumours, mainly originating in the tonsils, base of the tongue and elsewhere in oropharynx and occurring particularly in the Western world (10). It is now well accepted that oropharyngeal squamous cell carcinomas (OPSCCs) can be divided into HPV-negative (HPV–) and HPV-positive (HPV+) disease (11), with different prognostic outcome between the two subgroups (12). While HPV− cancers are believed to arise through a field cancerization process and a clonal progression in a setting of continuous carcinogen exposure, the HPV+ tumors harbor few mutations and are driven by a fundamentally distinct pathophysiologic mechanisms, which rely on E6 and E7 viral proteins to inactivate or bypass cellular tumor suppressive responses. Although recent vaccines against HPVs will influence the prevalence of HPV+ HNSCC in the decades to follow, for now, the incidence of HPV+ HNSCC continues to rise. Current estimates suggest that 45-90% of OPSCC are HPV+ with 90% associated with HPV genotype 16. HPV+ and HPV- tumours represent different clinicopathological and molecular entities with widely disparate survival rates.

1.1.3 Staging

The prognosis for patients with HNSCC and the treatment choice is largely determined by the stage of tumor at presentation. Tumors are staged according to tumor-node-metastasis (TNM) classification system, which takes in account the size of primary tumor (T), the nearby lymph nodes involvement (N) and the presence of distant metastasis (M). According to the last edition of the American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC), a new TNM classification became effective on 1 January 2018. This new version accounts for the importance the tumour depth of invasion in oral cavity tumors and has a different staging system for p16INK4A-positive tumours of the oropharynx, where p16INK4A positivity is used as a surrogate marker for human papillomavirus (HPV) status, which results in a lower stage for these tumours than that assigned by the previous edition (13) (Figure 4).

Seventh edition TNM	Eighth edition TNM
Stage I (T1N0)	Stage I (T1–T2N0–N1)
Stage II (T2N0)	Stage II (T1–T2N2 or T3N0–N2)
Stage III (T3N0 or T1–T3N1)	Stage III (T4 or N3)
Stage IVa (T4aN0–N1 or T1–T4aN2)	Stage IV (M1)
Stage IVb (T4b or T1–T4bN3)	–
Stage IVc (M1)	–

Figure 4. TNM Classification system (Seventh and Eighth editions) in HNSCC. Leemans et al., 2018.

In general, early stage (I or II) HNSCC is treated with local therapy, taking advantage of the ability of surgical removal and/or radiation. Advanced disease (stage III or IV) requires multimodality treatment with surgery, radiation, and/or chemotherapy.

1.1.4 Clinical presentation

It is possible to identify precancerous lesions in the mucosal linings that can look white (leukoplakia) or red (erythroplakia) areas of the mucosa. These lesions may progress and develop into invasive cancers (14). Leukoplakia is the most common precursor lesion of OPSCC (15). Risk factors for progression are female gender, size of lesion and the presence and grade of dysplasia (16).The clinical management is to treat the lesion, when possible, and analyse the histology of biopsy sample to understand the presence of dysplasia and the grading (mild, moderate or severe), which associates with cancer risk (16) (17). Besides these macroscopically recognizable lesions, many HNSCC can appear as *de novo* tumours, clinically not visible, identifiable only under the microscope as dysplastic mucosal epithelium. These morphological abnormalities already led in 1953 to the concept of 'field cancerization', both to describe these large dysplastic changes, but also to explain the high frequency of tumour recurrence after HNSCC excision and the high risk of multiple independent tumours developing in the mucosal linings (18). Slaughter et al. studied these precancerous changes with genetic markers, showing that the number of genetic alterations correlates with the severity of the dysplastic changes. In particular, the loss of heterozygosity at chromosomes 3p, 9p and 17p seemed to occur in dysplasia, apparently reflecting early carcinogenesis, whereas other alterations at chromosomes 11q, 4q and of chromosome 8 were typically present in carcinomas. Using these genetic markers combined with TP53 mutations, it was shown that in at least 35% of the oral and oropharyngeal tumours analysed, the carcinoma was surrounded by mucosal epithelium that contains genetic changes (19). In retrospective studies, it was shown that these unresected fields are an important source of

the local recurrences and the second primary tumours that are so often seen in patients with HNSCC (20) (21) (22). Comparison of the genetic profiles of carcinomas and their surrounding fields often indicates a clonal relationship (23) and this idea represented the basis of the hypothesis that such a field of contiguous preneoplastic cells precedes the development of an invasive carcinoma (24). Most recent studies have identified genetic changes at chromosome arms 3p and 9p, together with mutations in TP53 (residing at 17p) as the best predictors of malignant transformation in both leukoplakia (25) and surgical margins (26).

1.1.5 Therapeutic approaches

Treatment choice for HNSCC is based on the anatomic site of the tumor and TNM staging. Early stage (I or II) HNSCC is treated with surgery and/or radiation therapy. Advanced disease (stage III or IV) requires a multimodality treatment with surgery, radiation, and/or chemotherapy (27). Surgery remains the mainstay of early stage HNSCC. Small lesions are easily accessed transorally, while large tumors may require mandibulotomy, a more invasive approach. Due to the anatomic complexity of the oral cavity and the crucial role that it plays in taste, swallowing, speech and breathing, reconstructive decisions must take into consideration and are associated with functional and cosmetic outcomes. Advanced stage cancers are treated with combination therapy, usually surgical resection followed by adjuvant radiation with or without chemotherapy. Evidences, supporting the use of postoperative adjuvant chemotherapy with cisplatin versus radiation alone for HNSCC, came from two randomized trials published in 2004. Independently, studies designed by the European Organization for Research and Treatment of Cancer (EORTC 22931) and the Radiation Therapy Oncology Group (RTOG 95-01) demonstrated improved locoregional control and disease-free survival among HNSCC patients, who received postoperative chemoradiation therapy, rather than radiation alone (28) (29). More recently, a study looking specifically at oral cavity squamous cell carcinoma (OCSCC) showed that the addition of chemotherapy to radiation, after surgery, in advanced stage tumors led to a significant increase in 2- and 5-year overall survival compared to adjuvant radiation alone (30). Currently, chemoradiotherapy for patients with oral cavity cancer is limited to those with non-resectable tumors or comorbidities precluding surgery. Since the publication of a phase III clinical trial by Vermorken et al. in 2008, the combination of cetuximab, cis- or carboplatin and 5-fluorouracil has been the standard of care for patients with recurrent or metastatic HNSCC (31). More recently, the PD-1 (Programmed cell death protein 1)

antibody nivolumab and pembrolizumab have been approved for the treatment of patients with platinum-resistant disease (32). Notwithstanding improvement of radiation and surgical techniques and the addition of chemotherapy and monoclonal antibodies in advanced disease more than half of all patients experience relapse of disease, not suitable for treatment options with curative intention (33).

1.2 Cisplatin

1.2.1 Structure and mechanism of action

Cisplatin (cis-Diamine dichloroplatinum (II)) or cis-DPP (CDDP) is the most widely used cytotoxic drug for HNSCC. CDDP is a neutral, square planar molecule of platinum (II) bound to two chloride and two ammonia groups, where the chloride molecules are in the cis-geometry (34) (Figure 5). Cisplatin drug is able to diffuse into tissues and to bind primarily to plasma proteins. Due to the strong reactivity of platinum against sulphur and thiol groups of amino acids such as cysteine, nearly 90% of platinum in the blood is bound to albumin and other proteins leading to the inactivation of the majority of cisplatin.

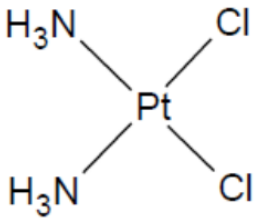


Figure 5. Structure of cisplatin.

The loss of chloride groups is required for the binding to genomic DNA (gDNA). Interestingly only 10% of the covalently bound cell-associated cisplatin is found in the gDNA and about 75-85% of the drug binds to proteins, RNA, thiol-containing peptides, and other cellular components rich in nucleophilic sites such as cytoskeletal microfilaments (35). The formation of the crosslinks inhibits DNA replication and transcription, by stalling the replication machinery at the site of the crosslink that can bend the double helix towards the major groove or even unwind it (36). Recognition of the damage, failure to unhook the crosslink, replication machinery stall, indirect further damage produced by the crosslinks and activation of other signalling pathways contribute towards cisplatin cytotoxicity (37) (38) (39). Then, cytotoxicity produced by CDDP has been shown to be a consequence of DNA damage caused by the formation of CDDP-DNA adducts. A major limitation in the

clinical use of CDDP is the acquisition of resistance of the initially responsive tumors (38). Resistance to CDDP treatment may be due to several mechanisms, including altered cellular drug transport, enhanced intracellular detoxification, increased DNA repair, adduct tolerance and modulation of apoptosis (38). CDDP activates nuclear as well as cytoplasmatic signaling pathways involved in regulation of the cell cycle, damage repair and programmed cell death. Understanding the cellular responses to CDDP is critical for determining mechanisms of drug resistance and for the development of therapeutic approaches for increasing the effectiveness of CDDP or other anticancer drugs that act by similar mechanisms. In particular, it has been reported that CDDP activates a membrane-integrated protein, the epidermal growth factor receptor (EGFR) (40). There are at least three potential mechanisms by which EGFR might interact with chemotherapy: through the cell cycle, through EGFR signalling or through DNA repair.

1.2.2 EGFR pathway activation upon CDDP treatment

EGF receptor is a 170 kDa transmembrane glycoprotein tyrosine kinase receptor, resulted overexpressed in about 90% of HNSCC. EGFR is composed by an extracellular ligand-binding domain (621 amino acids) and an intracellular protein tyrosine kinase domain (542 amino acids) connected by a small transmembrane-anchoring region (23 amino acids) (41). Within the broad family of receptor tyrosine kinases, EGFR belongs to the type I subfamily (or ERBB tyrosine kinase receptors) that also includes ERBB2 (also known as HER2 and NEU), ERBB3 (also known as HER3) and ERBB4 (also known as HER4). The binding of a ligand, such as EGF, transforming growth factor-α (TGFα) or amphiregulin causes the EGFR to dimerize with itself or with another member of the ErbB family of receptors, leading to receptor-linked tyrosine kinase activation and the activation of downstream signalling cascades that are crucial for normal cell growth and proliferation. Because aberrant EGFR activation has been associated with uncontrolled cell proliferation, EGFR is an attractive and logical target for cancer therapy. EGFR phosphorylation occurs in response to various cytotoxic drugs, including gemcitabine (42), cisplatin (40), oxaliplatin (43), 5-fluorouracil (43), paclitaxel (44), doxorubicin (45) and irinotecan (46). The phosphorylation of EGFR by oxaliplatin or 5-fluorouracil treatment alone correlates with the synergistic inhibition of cell viability and cell growth by gefitinib (43). The blockade of EGFR activation in response to paclitaxel by gefitinib promotes apoptosis and suppresses tumour growth (44). At the same time, one must consider the context in which EGFR phosphorylation occurs. It has long been known that EGFR phosphorylation occurs after

exposure to normal ligands such as EGF, which leads to cell growth and, ultimately, to physiological receptor degradation after proliferation is completed (47). The mechanism by which this chemotherapy-induced EGFR phosphorylation occurs is unknown. It has been proposed that EGFR signalling might serve as a cell-survival response in cells exposed to cytotoxic stress (48). The activation of EGFR in response to cisplatin occurs in an Src-dependent manner (40). The inhibition of EGFR activation (directly or by Src inhibition) increases cisplatin induced cell death, indicating that EGFR activation by cisplatin is a survival response. EGFR phosphorylation results in the activation of signalling cascades that activate Akt, which is a known anti-apoptotic kinase, probably indicating an EGFR-mediated survival mechanism. By blocking this survival signal, EGFR inhibitors could increase the effectiveness of chemotherapy and result in synergistic cytotoxicity.

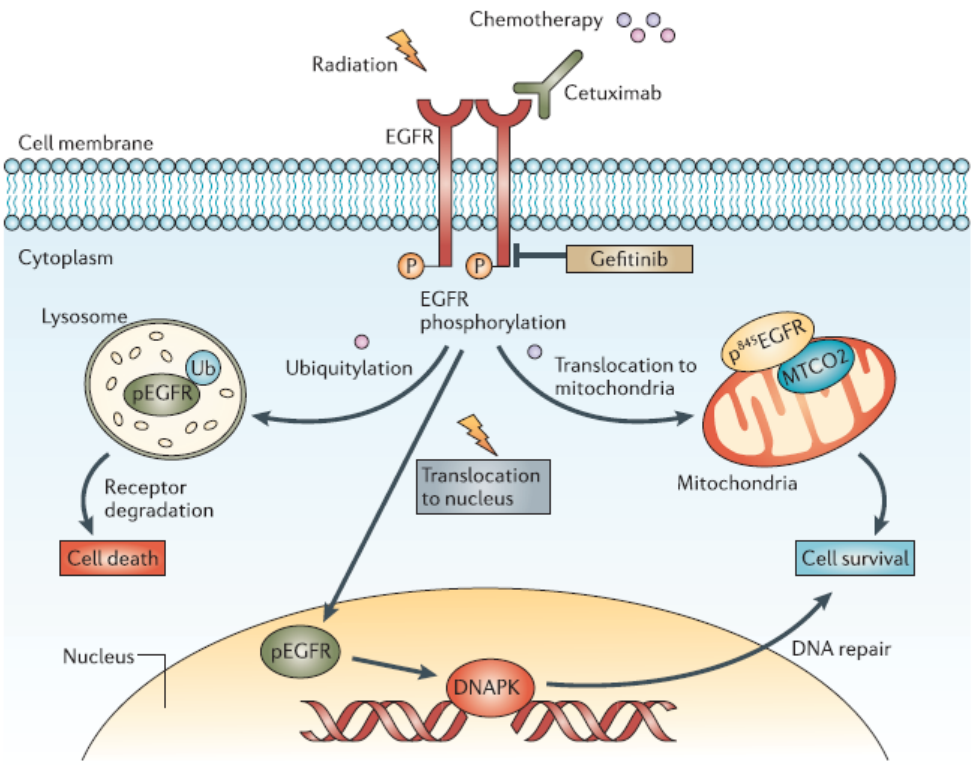


Figure 6. The effect of radiation and chemotherapy on EGFR signaling. Nyati et al., 2006.

1.3 Neuropilins (Nrps)

1.3.1 Structure

Neuropilins (Nrps) are 130-140 kDa single-pass transmembrane proteins involved in development, cancer and immunity. Nrps have been originally described as high-affinity

receptors for axon guidance molecules, such as class 3 semaphorins (SEMA3s) and subsequently as coreceptors for several members of the vascular endothelial growth factor (VEGF) family. Moreover, growing evidences have showed the role of Nrps in interaction with other transmembrane receptors molecules, enhancing the response to several growth factors (EGF, FGFs, HGF, PDFG, TGFs) and other mediators (integrins). Although the importance of Nrps in axonal guidance and angiogenesis is established, their role in cancer needs investigations. Nrp-1 was the first member of the Nrps family to be described in 1987 and Nrp-2 was isolated later by Chen et al. in 1997, by RT-PCR and gene transfer (49) (50). In humans, NRP-1 and NRP2 genes map to two different chromosomes, chromosomes 10p12 and 2q34, respectively (51). Although Nrps share only 44% homology in their amino acid sequences, some similarities can be observed in their structure. Nrps are composed by an extracellular domain, a transmembrane domain and a short intracellular domain (Figure 5). The extracellular region contains two complement-like binding domains (a1 and a2 domains), two coagulation factor V/VIII homology-like domains (b1 and b2 domains) and a meprin-like domain (c domain) (52). The single transmembrane portion is followed by a short cytoplasmic tail, terminating with a consensus sequence, able to interact with PDZ (PSD-95/Dlg/ZO-1 homology) domain proteins. Extracellular "a" and "b" domains are implicated in ligand binding, in particular SEMA3s bind to the a1/a2/b1 segment and VEGFs binds to b1/b2. "c" domain mediates Neuropilin homo- and heterodimerization, which seems to be essential for function.

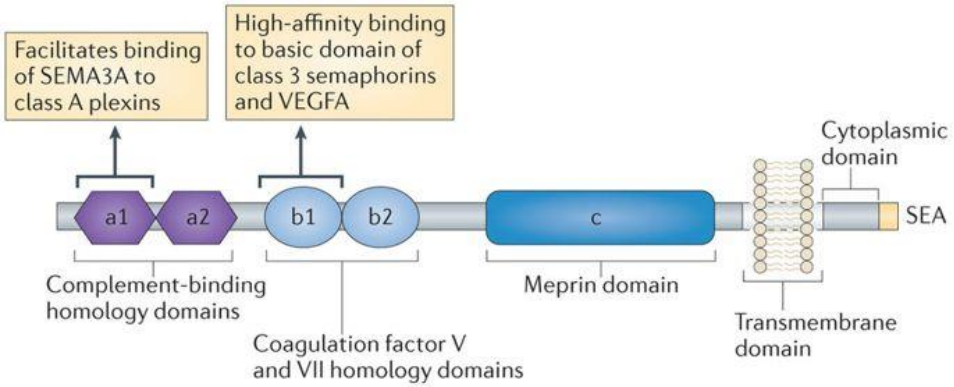


Figure 7. Schematic representation of Nrps structure. Kumanogoh et al. 2013.

There are contradictory data on the signaling competence of the small conserved cytoplasmic tail of neuropilins. According to many reports, Nrps essentially provide a ligand-binding platform, while intracellular signalling is mediated by associated plexins or VEGF receptors (VEGFR). Other findings, however, suggest an independent signaling function of the

intracellular domain of neuropilins. For instance, a cytosolic Nrp-1-interacting scaffold protein, GIPC (also known as synectin), was involved in Nrp-1-dependent function in angiogenesis. Thus, by interacting with GIPC and potentially additional PDZ domain-containing proteins, Nrps could regulate receptor complexes in the plasma membrane. Notably, the cytoplasmic tail of Nrp-1 and Nrp-2 are largely divergent, which raises major questions on whether they may interct with different adaptors or signal transducers.

1.3.2 Nrps Function in Semaphorin dependent axon guidance and VEGF dependent angiogenesis

Nrps were originally identified as co-receptor for class 3 semaphorins, a family of molecules that provide repulsive or attractive signals for neurons [47-48]. In particular, it has been showed Nrps involvement in neural crest migration and axon growth during the development of the nervous system by forming a complex with type-A plexin, a signal-transducing transmembrane receptor for class 3 semaphorins (53) (54). Subsequently, Nrps were identified as receptors for VEGF. NRP-1 is expressed in endothelial cells, where it interacts with several members of VEGF family and some of their tyrosine kinase receptors, enhancing the signalling and promoting angiogenesis. In particular, Nrp-1 signaling is critical for VEGF-A/VEGFR-2-mediated angiogenesis (55) and Nrp-2 signaling is important for VEGF-C/VEGFR-2/R-3-mediated lymphangiogenesis (56) (57). Nrp-2 is highly expressed in lymphatic tip cell filopodia and selectively modulates VEGF-C/VEGFR-3-mediated tip cell extension (58) (59). Although the signaling pathways for Nrp-1 and Nrp-2 are largely distinct, they are able to partially compensate for each other in certain biological contexts, and the double knock-out mice of NRPs genes (NRP-1−/− NRP2−/−) has a more severe phenotype than the single knock-out mice (NRP-1−/− or NRP2−/−), with impairing any blood vessel development and causing earliest death in utero at E8.5 (60). Nrps present differences in their expression patterns, ligands specificities and signalling pathways.
In this study we focalize our attention on Nrp-1.

1.3.3 Neuropilin-1

In addition to its established function as cell surface coreceptor for Plexins and VEGF receptors, Nrp-1 has been found to interact with many other transmembrane receptor molecules (such as EGFR, MET, IGF1-R, PDGF-R tyrosine kinases, TGFβ receptors,

integrins, etc.) and elicits a range of intracellular signalling cascades. Consequently, Nrp-1 is implicated in the mediation of several cellular processes, such as proliferation, survival, invasion and migration. Nrp-1, as a primary function, promotes pathway activation by recruiting ligands to the cell surface through high-affinity interactions. Ligand binding is followed by the assembly of active signalling complex, where Nrp-1 functions in promoting and stabilizing the complexes (61). The assembly of the active signalling complex requires both receptor recruitment and binding, followed by associated conformational changes, which provide the physical mechanism required for signal transduction. Moreover, upon the assembly of the active signaling complex, Nrp-1 has been demonstrated to have a critical function in receptor trafficking. Intriguingly, it was shown that Sema3 and VEGF induce Nrp-1 endocytosis via distinct pathways (62). Furthermore, Nrp-1 promotes the partitioning of VEGFR into vesicles that are recycled back to the cell surface, whereas in the absence of Nrp-1, VEGFR2 is targeted for degradation instead of recycling (63). The intracellular GIPC-binding domain of Nrp-1 was demonstrated to be essential for the observed VEGFR recycling, suggesting the involvement of a Nrp-1 associated cytoplasmic protein. While a growing body of evidence supports the relevance of Nrp-1 in tumor growth and malignant progression, the implicated molecular mechanism are still to be explored.

1.3.4 Nrp1 expression in human tumors and other tissues

Nrp-1 is widely expressed in a variety of cancer cell lines and human tumors, while usually low or absent in the adjacent non-malignant counterpart. Clinicopathological data often indicate a correlation between increased expression of Nrp-1 and advanced stage tumors with poor prognosis. For instance, high Nrp-1 expression levels correlate with poor clinical outcome in patients with non small cell lung cancer, melanomas, pancreatic carcinoma and oral squamous cell carcinoma (64) (65) (66) (67). Moreover, it has been reported also the correlation of Nrp-1 expression with invasive behaviour and metastatic potential in gastric cancer, glioma, prostate carcinoma and melanoma (68) (69). In addition, Nrp-1 expression occurs also in the tumor microenvironment, such as in endothelial cells of tumor vessels, as well as in tumor-associated macrophages (TAM), and regulatory T lymphocytes (Treg) (70). In these cell types, Nrp-1 was also reported to support tumor development, and its cell-specific genetic depletion in mice led to reduced tumor vasculature and tumor growth, defective TAM recruitment and increased anti-tumor immune response (71) (72). The mechanism driving Nrp1 overexpression in tumors is still poorly understood but it supports the relevance of the molecule in malignant progression.

1.3.5 Nrp-1 and EGFR

EGFR pathway is frequently activated in human tumors and pivotally implicated in sustaining cell proliferation. This may be associated with receptor gene amplification and overexpression or ligand overexpression and autocrine signaling in cancer cells. Constitutive EGFR activation sustained by autocrine TGF-a signaling was strikingly dependent on Nrp1 expression in cancer cells. It has been showed that Nrp-1 play the role of EGFR co-receptor. Nrp-1 can physically interact with EGFR on the cell surface and this association is induced by EGFR ligands, EGF and TGF-a (73). Subsequently the ligand stimulation, there is the formation of receptor clusters, dependent on Nrp-1 expression. EGFR oligomerization and clustering is followed by internalization and signalling in endosomal compartments, especially relevant for enhancing Akt activity (Figure 8A). It is well known that receptor endocytosis is a major regulatory mechanism controlling receptor signaling in space and time. This is particularly important for EGFR, because the signaling cascade elicited by receptor activation is not limited to the plasma membrane, and crucially continues during receptor trafficking through endosomal compartments, especially for mediating AKT activation. Ligand-induced EGFR internalization into endocytic vescicles is impaired in the absence of Nrp-1 and strikingly attenuates Akt phosphorylation (Figure 8 B) (73). Upon Nrp-1 depletion, EGFR signaling is significantly affected in cancer cells with the impairment of viability and proliferation in a variety of cancer cell models (Figure 8C) (73).

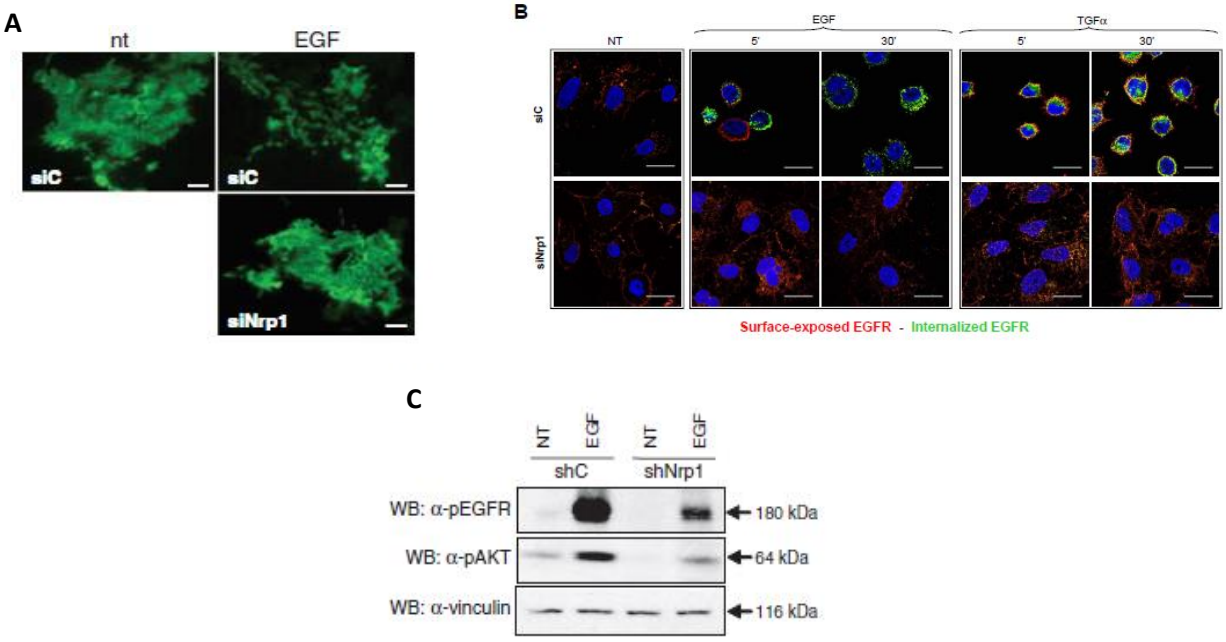


Figure 8. A) Nrp-1 regulation of ligand-induced EGFR clustering on the cell surface. B) Ligand-induced EGFR internalization is dependent on Nrp1. C) Nrp-1 regulation of ligand-induced EGFR signaling. Rizzolio et al. 2012.

CHAPTER II

-AIM OF THE STUDY-

2. Aim of the study

In recent decades, despite therapeutic advances in the HNSCC treatment, patient survival has not markedly improved and the mortality still is around 40-50 %. The limited informations available on the biology of HNSCC claim an urgent search for novel biomarkers in order to design new therapeutic strategies. Nrp-1 results an interesting candidate to explore in HNSCC, on account of its role as co-receptor of EGF receptor.

To address this issue, we adopted two different models.

First, we took advantage of a study population of HNSCC tissue specimens, collected from 2000 to 2017, at the Pathology Section of the University of Naples "Federico II" to investigate whether:

1) The IHC Nrp-1 expression correlated with the clinicopathological features of the patients and with prognosis;

Then, by using *in vitro* HNSCC cell models, in which we have achieved stable Nrp-1 depletion, we explored whether:

2) The Nrp-1 depletion could impact the sensitivity to cisplatin drug;
3) The Nrp-1 depletion could modulate the cisplatin-induced EGFR activation.

Overall, the sum of these investigations let us hypothesize a new targeted therapeutic approach for the HNSCC treatment.

CHAPTER III

-MATERIAL AND METHODS-

3. Materials and methods

3.1 Cell culture and culture conditions

The HN, CAL27, CAL33, HN6 and HN13 cell lines were provided by Angela Celetti of the Institute of Experimental endocrinology and oncology-C.N.R. of Naples. They were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (Sigma-Aldrich, UK), 1% of 200mM L-glutamine (Autogen Bioclear, UK), 1% of 10,000 units Penicillin –10mg/ml Streptomycin (Sigma-Aldrich, UK) and incubated at 37 °C in 5% CO2.

3.2 Drugs

Cis-Diammineplatinum(II) dichloride (cisplatin) (C2210000) was purchased from Sigma-Aldrich S.r.l. and Saracatinib (AZD0530) from Selleckchem.

3.3 Cell viability

For in vitro viability assays, cells were seeded (1000 cells/well, three technical replicates) and after 24 h treated with different cisplatin doses (2,5; 5; 10; 20 μM) or vehicle (Phosphate-buffered saline, PBS) for 72 h. After the end of treatment, cell medium was discarded, the CellTiter-Glo reagent (Promega Inc, Madison, WI, USA) was added and the plate was incubated for 10 min at room temperature. Luminescence was measured in a Multilabel Reader (PerkinElmer, Waltham, MT, USA).

3.4 Gene silencing

To achieve stable knockdown NRP1 expression was silenced in tumor cells by transducing cells with shRNA-expressing lentiviral constructs. NRP1-targeting sequence (GAGAGGUCCUGAAUGUUCC) was inserted in the lentiviral transfer plasmid pCCLsin.PPT.hPGK.GFP.Wpre in the frame of a sequence driving the transcription of a short-hairpin RNA under control of the H1 promoter. Control shRNA (shC) was generated by introducing 4 base substitutions in the NRP1 targeting sequence (GATAGGTCATGACTGCCC). We silenced NRP1 expression by means of a puromycin

selectable lentiviral construct TRCN0000323055, provided by Sigma-Aldrich; plkO vector was used as control.

3.5 Western blot analysis

Whole-protein extracts were prepared using LB buffer (½ vol. H2O, ¼ vol. Tris HCl pH 6.8, ¼ vol. sodium dodecyl sulphate 10%) and quantified using the BCA Protein Assay kit (Pierce, Rockford, lL, USA). Primary antibodies: anti-Nrp1 (ab81321) and antibody against phosphorylated EGFR (Tyr1068) (ab5644) was from Abcam (Cambridge, UK); anti-EGFR (1005:sc-03), phosphorylated ERK (Thr202/Tyr204), phosphorylated AKT (Ser473) (Clone D9E), total AKT, and ERK were from Cell Signaling and antibodies against Vinculin (1931) from Sigma. Secondary antibodies were from Amersham. Detection was performed with ECL system (Amersham, UK).

3.6 RNA isolation and real-time PCR

Total RNA from tumor cell lines or tissues was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA preparation was done according to standard procedures, using M-MLV Reverse Transcriptase (Promega) and oligo-dT primers (Promega). Gene expression was measured using the following Taqman gene-specific probes from Thermo Fisher Scientific: NRP1 (Hs00826128_m1), EGFR (Hs00193306_m1), and the housekeepers GAPDH (Hs04420632_g1) and β-actin (Hs99999903_m1).

3.7 Patients and tumor samples

Head and neck tumors and matched normal samples were obtained from patients undergoing surgery at the Department of Oral e Maxillofacial Surgery of the University of Naples "Federico II". Tissue samples were collected, handled and diagnosed at the Pathology section of the same University. Clinical and pathologic data were entered and maintained in our database. The study design and procedures involving tissue samples collection and handling were performed according to the Declaration of Helsinki, in agreement with the current Italian law and to the Institutional Ethical Committee guidelines.

3.8 Tissue Micro Array (TMA)

The tissue microarrays were carried out by the assembly of tissutal cores (1-3 mm), selected from every sample and built in recipient paraffin blocks. The TMAs were set up selecting two cores for each malignant tissue sample and one taken from the adjacent normal counterpart, where available. Hematoxylin and eosin (H&E) staining, used also for diagnosis confirmation, revealed the HNSCC architecture. TMA sections (5 μm thick) were stained by immunohistochemistry (IHC) for human NRP1.

3.9 Immunohistochemistry

Tumors were fixed in formalin for 16 h and then paraffin embedded. Sections were cut (5 μm) and immunohistochemical analysis was carried out. Briefly, sections were deparaffinized and hydrated. Antigen retrieval was performed using microwave (750 W), carrying out 3 cycle of 3 minutes in Cytrate buffer solution. Endogenous alkaline phosphatase was quenched using Levamisole. Primary antibody included Nrp-1 antibody from Abcam (Cambridge, UK). Diluted antibody was applied to the sections overnight and then detected using anti-rabbit reagent coniugated with AP Substrate (Thermo Fisher). Tissues were counterstained with hematoxylin, dehydrated, cleared, and coverslipped. Staining for Nrp-1 was ranked according to the intensity of staining (0= negative, 1=weak, 2=moderate, 3=strong).

3.10 Statistical analysis

Correlation between Nrp-1 immunohistochemically expression and HNSCC clinicopathological characteristics was asses through contingence analysis with Fisher exact test. Statistical analysis has been performed using SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

CHAPTER IV

-RESULTS-

4. Results

4.1 Study population

The HNSCC study population comprises 217 HNSCC human samples that have been collected from the 2000 to the 2017 at the Pathology section of Department of Advanced Biomedical Sciences, University of Naples “Federico II”. The HNSCC cohort of patients is composed by 119 male (54,8%) and 99 female (45,2%), with an age range of 29-90 years and an average age of 63,17 years. The clinicopathological features, comprising sex, age, anatomic site, grading, stage, TNM and follow-up, were obtained by clinical history of the patients. Tumours were staged according to tumour-node-metastasis (TNM) classification (74).

4.2 Nrp1 expression is an independent prognostic marker for HNSCC patients

Upon the assembly of the TMAs, including the whole study cohorts of HNSCC patients, we carried out Nrp-1 staining, by immunohistochemistry. Two pathologists double-blinded scored Nrp-1 expression determining an immunoreactivity score (Figure 9), as described in “Material and methods” section, and also identifying two type of signals, in the cytosol (Figure 10 A) and in the membrane (Figure 10 B).

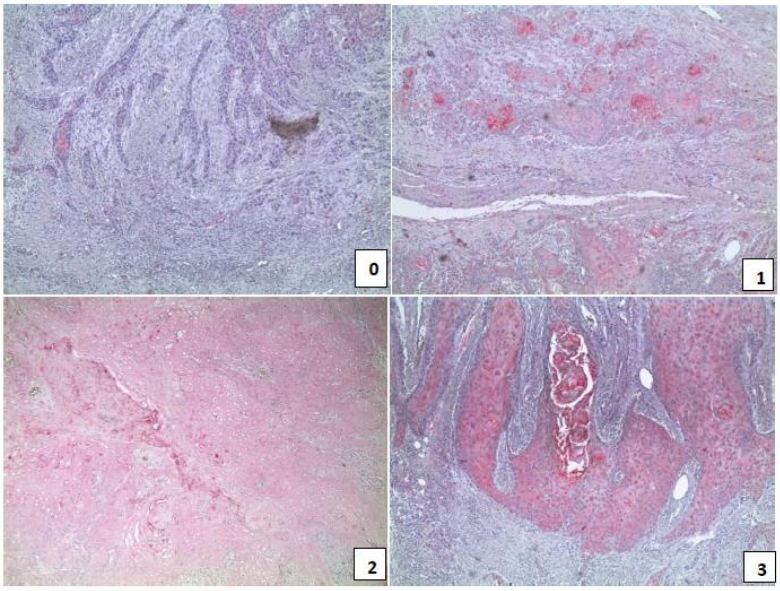


Figure 9. Representative pictures of IHC Nrp-1 expression in HNSCC specimens with the intensity score: 0= negative; 1= weak; 2= moderate; 3= strong.

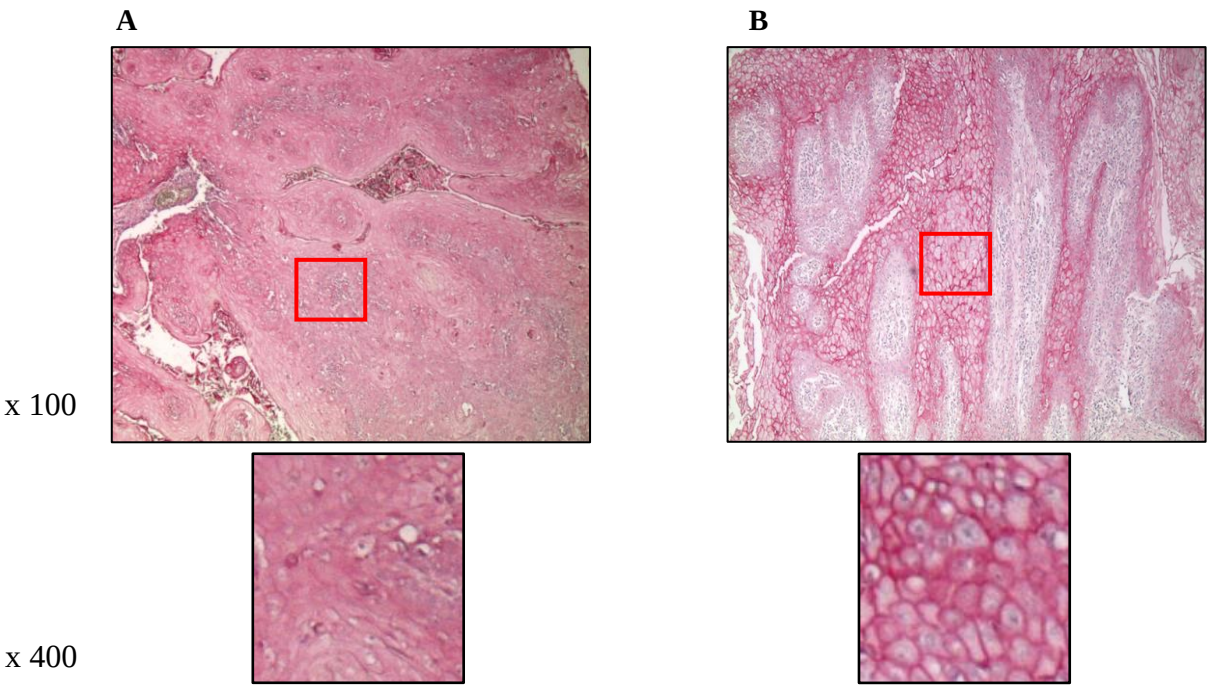


Figure 10. Representative images Nrp-1 staining localization (A. cytoplasmic, B. membranous) by IHC in HNSCC specimens.

On the basis of technical issues and of the clinicopathological features of the specimens, we were able to perform the analysis on N=116 non malignant tissue and on N=170 HNSCC malignant tissue samples. To perform data analysis, we divided the study population in two groups: a Nrp-1 low expression group, with score '0-1'and a high Nrp-1 expression group, with score '2-3'. In the non-malignant tissues, we observed a low Nrp-1 expression in 105/116 (90,5 %) of samples and a high Nrp1 expression in 11/116 (9,5 %) tissue samples. In the HNSCC samples, while we observed a low Nrp-1 expression levels in 157/170 (72,3%), we detected a high Nrp1 expression level in 36/170 (27,7%) cases. In order to perform the statistical analysis, we decided to take into considerations all the samples from study population with the follow up above 12 months. Considering the heterogeneity of the clinicopathological features of our HNSCC study population, we also divided the samples of the study population on the basis of the anatomic site, in oropharynx (OP) and in not oropharynx (NOP). Moreover, to better carry out the risk stratification, we clustered the samples of the study population according to the patients' age in three groups: patients with age under 40 years old, patients with a range in between of 41-59 years and patients over 60 years old. We performed the Kaplan Meier analysis considering both the cytoplasmic and of the membrane Nrp-1 positivity expression, in the three subgroups. Interestingly high Nrp-1 cytoplasmic expression significantly correlated with a shorter overall survival rate in the subgroup of NOP tumor patients, over 60 years old (p= 0,006) (Figure 11).

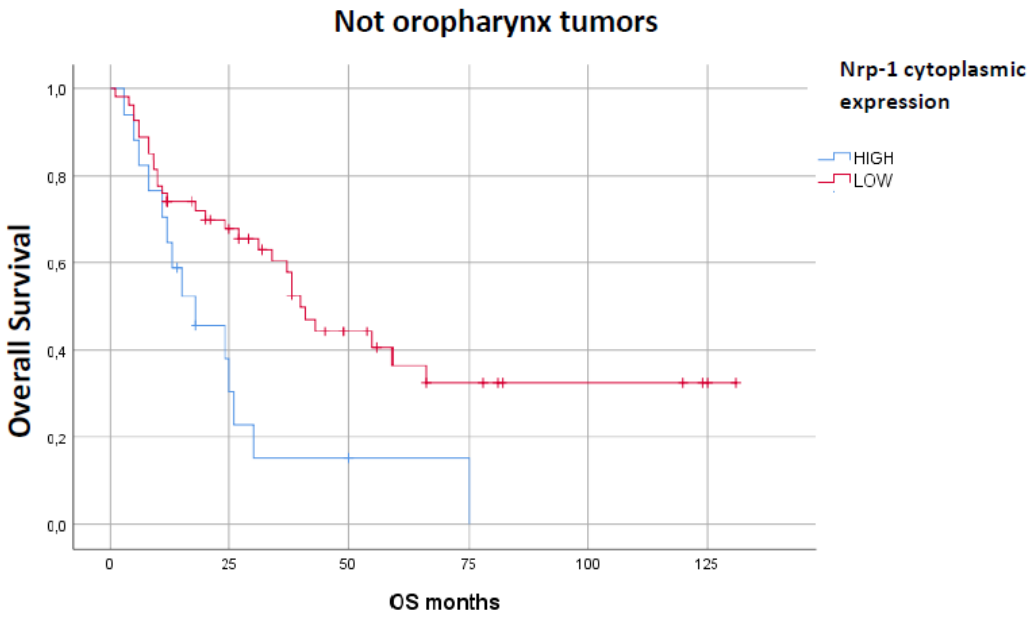


Figure 11. Overall survival analysis according to Nrp-1 cytoplasmic expression in not oropharynx HNSCC patients.

On the other hand, membranous Nrp-1 expression did not show any correlation with prognosis in the same subgroup of NOP patients (p=0.921) (Figure 12).

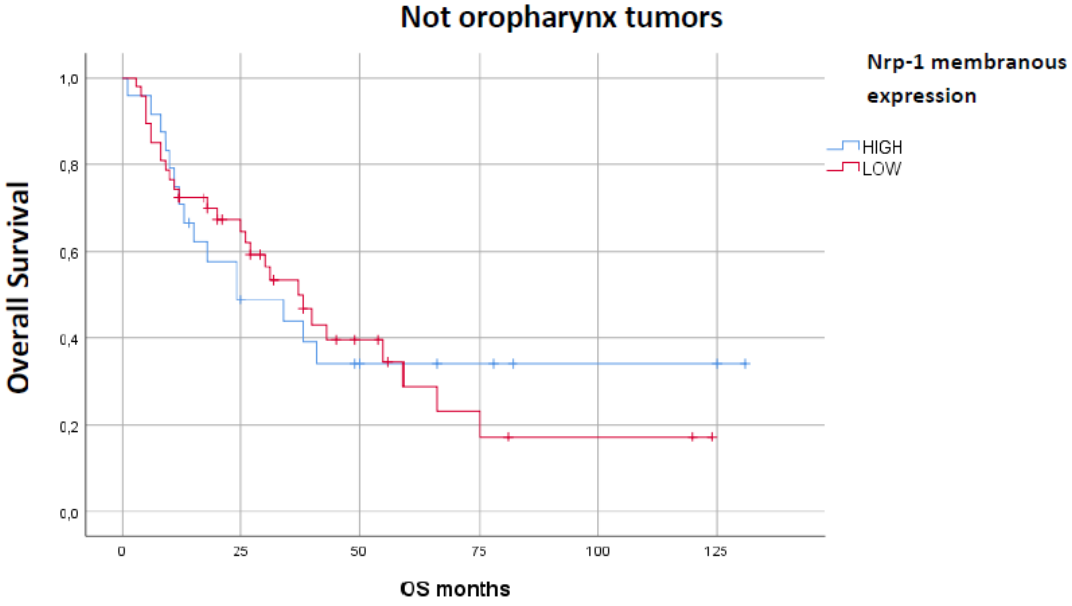


Figure 11. Overall survival analysis according to Nrp-1 membranous expression in not oropharynx HNSCC patients.

We performed the Kaplan Meier analysis taking into considerations all the clinicopathological features of this subgroup of patients. Besides Nrp-1, also the stage resulted to be of prognostic value in this group of clinical samples. Conversely, in OP tumors the stage and Nrp1 did not show any correlation with patient prognosis (p= 0,120), therefore we decided to follow on our analysis in this subgroup of study population samples as represented in the Venn diagram below.

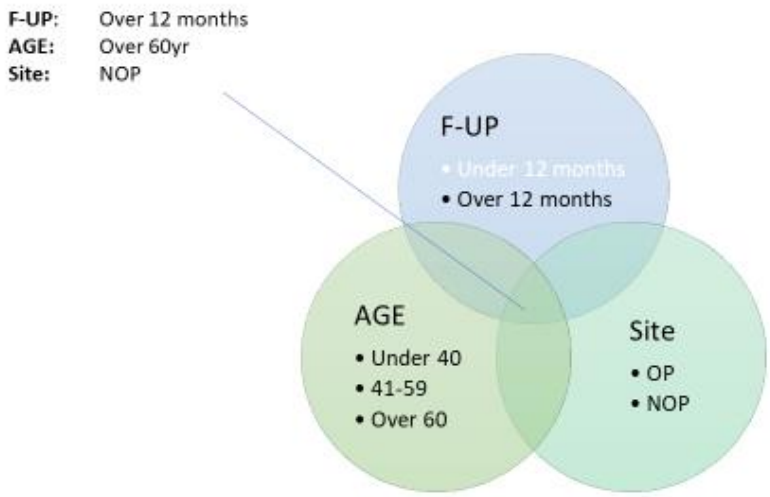


Figure 12. Venn diagram representing the features of the sorted study population (F-UP= Follow-up).

The sorting of the samples generated a novel study population composed by 78 patients, with an average age of 72,88 years and an age range of 60-90 years. In this group, we analysed the frequency distribution according to the tumor stage (Figure 13) and to the Nrp-1 localization (cytoplasmic and of membrane) (Figure 14).

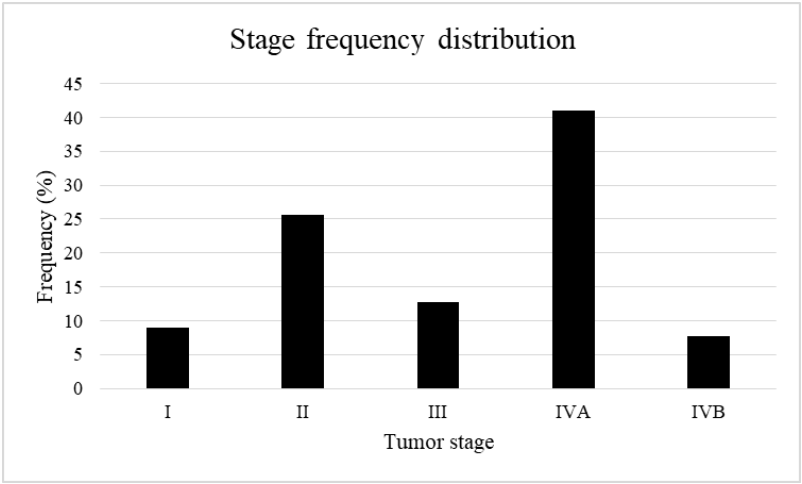


Figure 13. Stage frequency distribution in the HNSCC subpopulation.

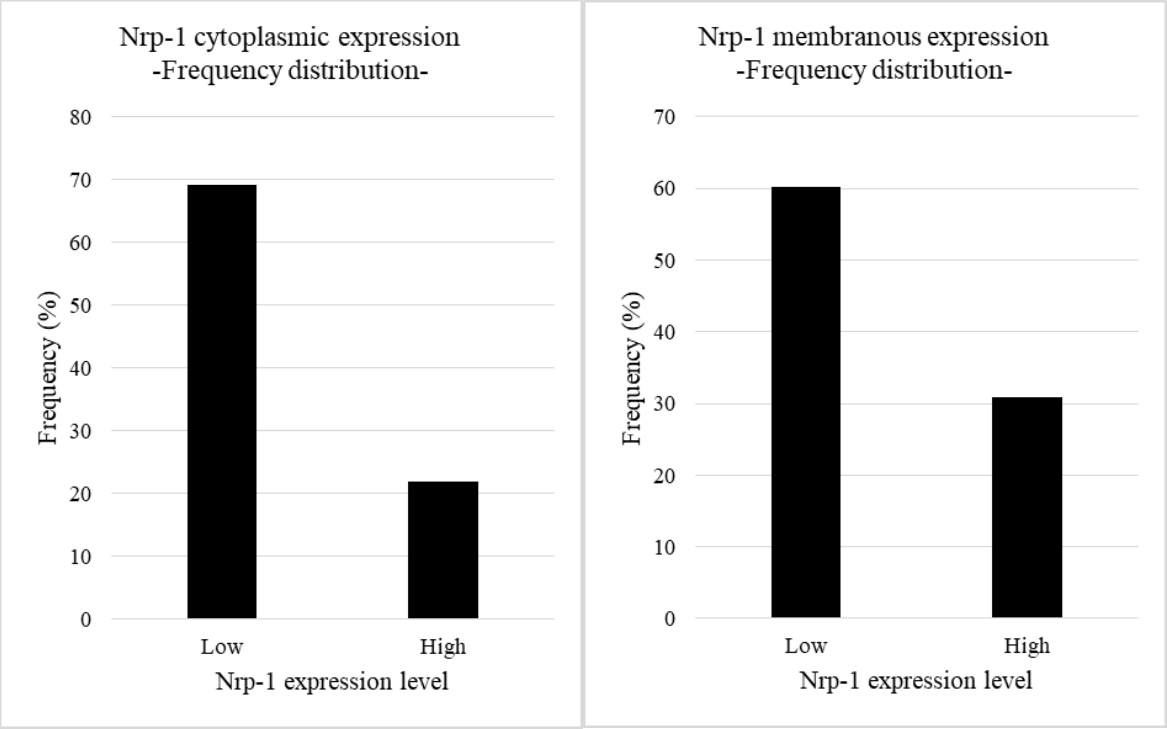


Figure 14. Frequency distribution of Nrp-1 cytoplasmic and membranous expression.

4.3 Nrp-1 expression level and localization allow risk stratification in a subgroup of HNSCC patients

Next, we performed a multivariate Cox regression analysis to understand whether Nrp-1 levels and the stage of disease could determine a stratification risk. Among the stage, only the tumors at the stage IV presented an Exp (B) value over 1. Noteworthy, we observed that the expression levels and cytoplasmic localization of Nrp-1 presented an Exp (B) value [1,455] (p=0,031) higher than the Exp (B) [1,081] (p= 0.917) of the stage, indicating, compared to the stage, Nrp-1 as a better prognostic factor for this subgroup of patients, characterized by NOP tumors in over 60 years old patients. By the schematic representation (Figure 15), it is possible to appreciate the model of regression analysis by cytoplasmic Nrp-1 expression. Taken together these data showed that Nrp-1 overexpression contributed to a shorter overall survival in a not oropharynx HNSCC subgroup of patients, with an age over 60 years old. In this study cohort, Nrp-1 expression and stage were independent prognostic factors for HNSCC patients, but Nrp-1 expression resulted to be a better prognostic marker than the stage.

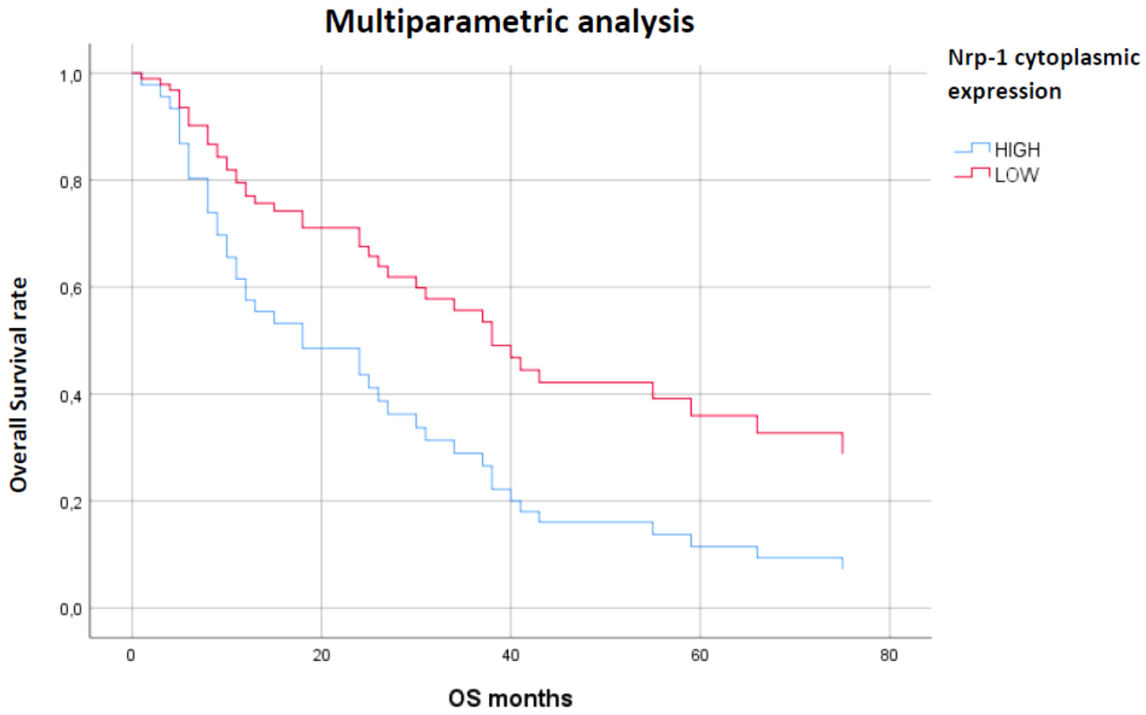


Figure 15. Regression analysis by cytoplasmic Nrp-1 expression.

4.4 Nrp-1, Nrp-2 and EGFR expression levels in HNSCC cell lines

Next, we moved in *in vitro* experimental models. We assessed in a panel of HNSCC cell lines (HN, HN6, HN13, CAL27, CAL33) the expression levels of Nrp-1, Nrp-2 and of EGFR, by Western Blot analysis (Figure 16). Nrp-1 resulted expressed in all the analysed cells. Nrp-2 was highly expressed in HN, while was detectable at low levels in CAL27 and CAL33 and resulted nearly absent in HN6 and HN13 cells. The levels of Nrp-2 resulted inversely correlated with Nrp-1 expression levels. To note, in HN6 and HN13 cells we confirmed high expression of EGFR, as already reported (75).

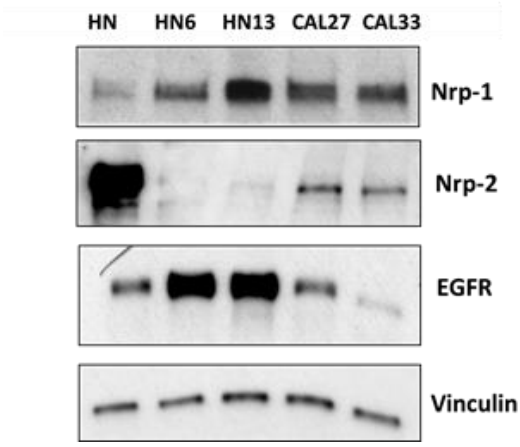


Figure 16. In the five indicated head and neck cancer cell lines, Nrp-1, Nrp-2 and EGFR relative expression levels are detected at Western Blot analysis.

In order to assess the Nrp-1 impact on cisplatin sensitivity we established the cellular models, by performing Nrp-1 knockdown in all the cell lines, as described in 'Material and methods' section. Nrp-1 silencing resulted detrimental in HN and CAL27 that did not survive upon silencing, suggesting that cell viability was dependent on Nrp-1 expression. Conversely, we obtained stable Nrp-1 knockdown in CAL33, HN6 and HN13. The wild type or mutant EGFR status of the analysed cells is shown in the Table 1.

Cell lines	EGFR mutational status	References
CAL33	wild type	(76)
HN6	amplificated	(75)
HN13	mutated (p.H773Y) and amplificated	(77)

Table 1. EGFR mutational status of the three HNSCC cell lines (CAL33, HN6, HN13).

Nrp-1 silencing efficiency and the EGFR expression levels were determined by Western blot (Figure 17) and Realtime-PCR (data not shown). Intriguingly, we noted that Nrp-1 silencing decreased EGFR expression in the HN6, which was downregulated through a negative regulation at transcriptional level (Figure 18).

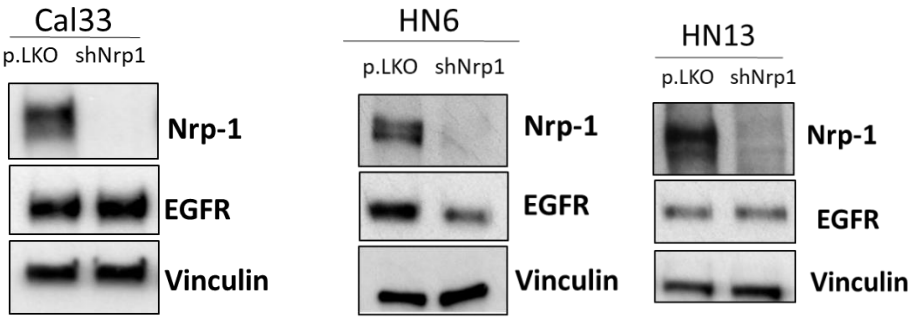


Figure 17. Nrp-1 and EGFR protein expression levels assessed in the HNSCC cells upon Nrp-1 silencing, by Western Blot analysis.

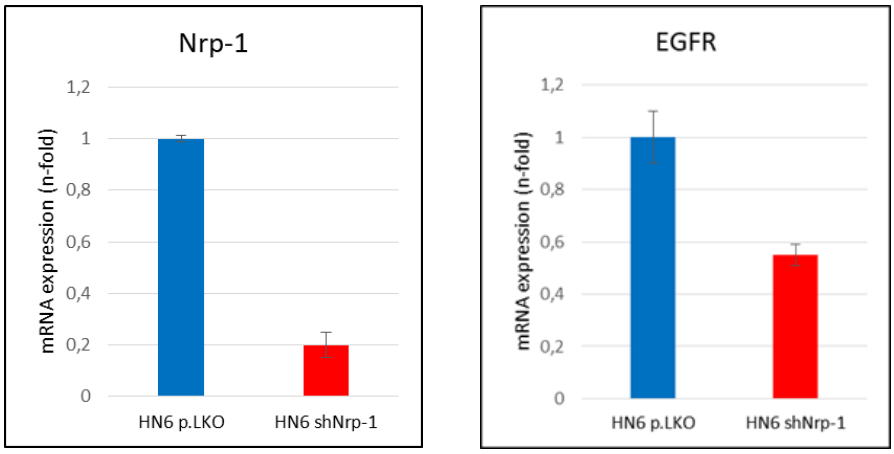
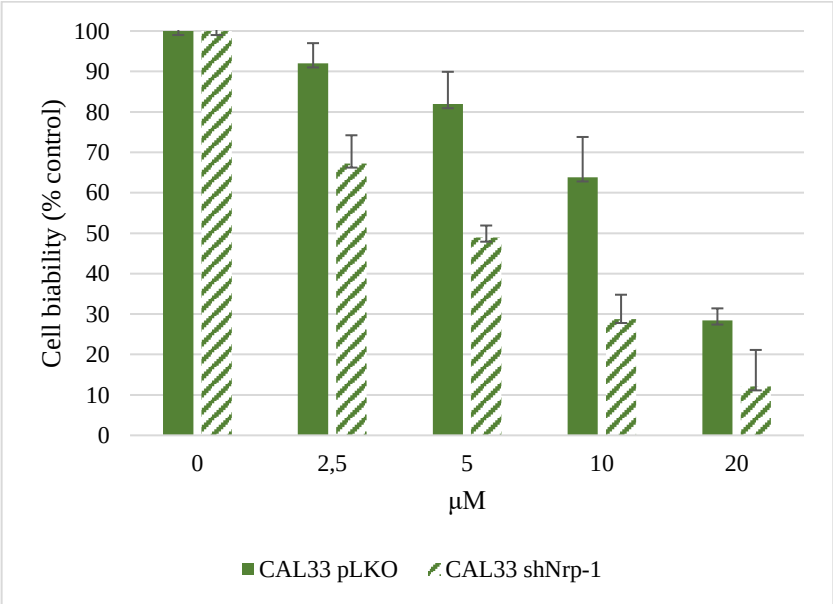
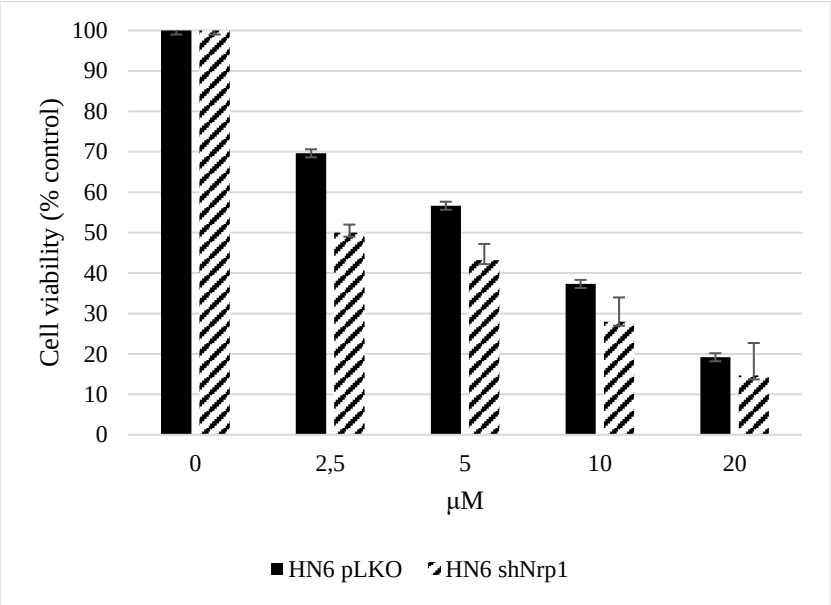


Figure 18. Nrp-1 and EGFR mRNA expression level (n-fold) in the HN6 shNrp-1 cells compared to HN6 pLKO, by Realtime-PCR.

4.5 Nrp-1 affects responsiveness to CDDP treatment

Next, we treated the Nrp-1 silenced HNSCC cells, compared to controls, with different CCDP doses (0; 2,5; 5; 10; 20 µM) for 72 hours and assessed the cells viability. Nrp-1 silencing determined an enhanced sensitivity to cisplatin in HN6 cells [shNrp1 IC50= 2,5 µM vs. plkO IC50 = 5,5 µM vs] and in CAL33 cells [shNrp1 IC50= 5 µM vs. p.LKO IC50 =12 µM] (Figure 19). However in HN13 cells, we did not appreciate a significant difference in the IC50 between the shNrp1 vs plkO cells, suggesting that Nrp-1 might have a major impact on CDDP sensitivity in the EGFR wild type (CAL33) and EGFR amplificated cells (HN6).





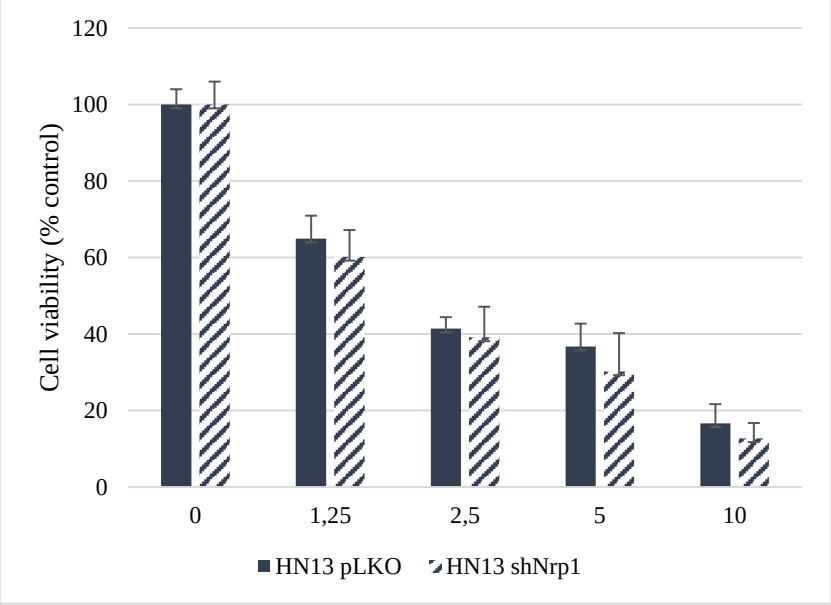


Figure 19. Cell viability of the Nrp-1 silenced HNSCC cells compared to the control, upon cisplatin treatment. Three independent experiments were performed to calculate the mean and standard deviation. The 50 % inhibitory concentration (IC50) was calculated for all the cell lines.

4.6 Nrp-1 sustains EGFR activation upon CDDP treatment

EGFR phosphorylation, besides of occurring in ligand dependent manner, may also take place in response to various chemotherapeutic drugs (CDDP, oxaliplatin, 5-fluorouracil, gemcitabine, doxorubicin). The mechanism by which chemotherapy-induced EGFR phosphorylation (p-EGFR) occurs is unknown but it may represent a cell-survival mechanism in response to cytotoxic stress. In order to determine whether Nrp-1 may be responsible of CDDP-induced EGFR activation, we performed a time course experiment, assessing EGFR tyrosine phosphorylation (Y1068) at 1, 4, 6 and 8 hours following CDDP treatment (50 μM) (Figure 9). A time-dependent EGFR phosphorylation was observed and the kinetics of receptor activation resulted to be different in the three cellular system, as shown in the Figure 20. In the HN6 Plko, the highest increase in the level of EGFR phosphorylation was achieved after 6 hours of CDDP treatment, with a consequent activation of the downstream effectors, AKT and MAPK. However, we appreciated a significant impairment of CDDP induced EGFR activation in the HN6 shNrp1 compared to HN6 plkO, even though the basal level of EGFR phosphorylation resulted equivalent in the two cell lines. No differences in EGFR, AKT and MAPK activation between the plkO and shNrp1 were observed in HN13 cells at all time points following CDDP treatment. On the contrary, in the CAL33 cells we detected a barely activation of EGFR at Western Blot analysis, and an impairment of AKT and MAPK activation. On the basis of these observations, we hypothesized a role for Nrp-1 in sustaining the CDDP induced cell-survival response also in the CAL33 cells.

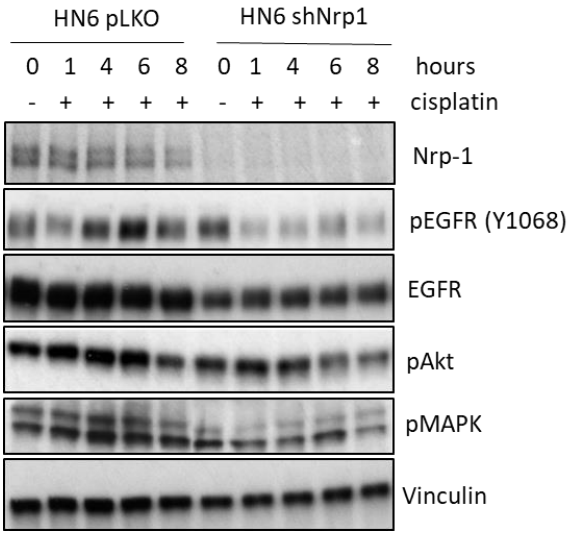


Figure 20. Time course of EGFR, AKT and MAPK activation in HN6 shNrp-1 cells compared to HN6 plkO cells, upon cisplatin treatment at different time points, assessed by Western Blot assay.

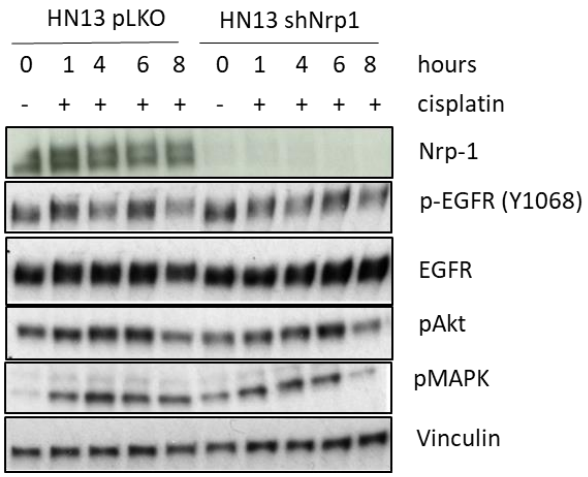


Figure 21. Time course of EGFR, AKT and MAPK activation in HN13 shNrp-1 cells compared to HN13 plkO cells, upon cisplatin treatment at different time points, assessed by Western Blot assay.

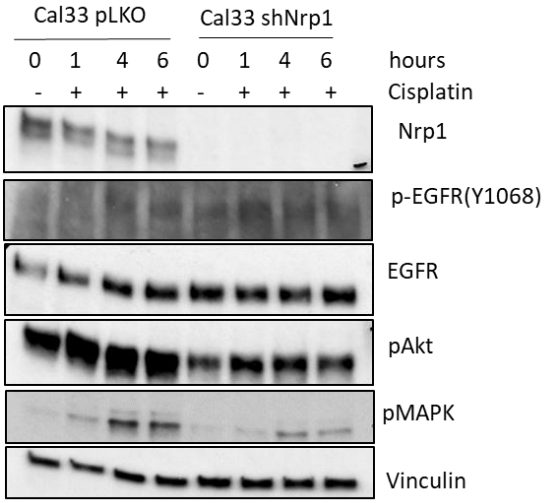


Figure 22. Time course of EGFR, AKT and MAPK activation in CAL33 shNrp-1 cells compared to CAL33 plkO cells, upon cisplatin treatment at different time points, assessed by Western Blot assay.

4.7 Effect of conditioned medium from CDDP treated NRP-1-expressing cells

Since in the HN6 plkO, Nrp-1 expression resulted in CDDP-induced EGFR phosphorylation enhacement, we sought to determine whether this effect could be dependent on secreted factors able to elicit autocrine pathways. To test this hypothesis, conditioned medium (CM) from HN6 plkO cells was collected upon CDDP treatment, after 6 hours, the time point of the highest EGFR phosphorylation. Then, by adding the CM, we performed a time course in HN6 shNrp-1 cells. We also carried out a time course in the HN6 plkO and shNrp-1 without

adding the CM, as control. At the different time points, we did not appreciate any difference in the activation of EGFR, following the addition of conditioned medium in the HN6 cells depleted of Nrp-1 upon treatment with CDDP. On the other hand, as previously observed, the HN6 plkO presented higher levels of EGFR phosphorylation compared to the HN6 shNrp-1, in both the condition (CDDP alone or in presence of CM). These findings suggested that the increase of EGFR phosphorylation observed in NRP-1-expressing cells was not elicited by the induction of autocrine factors production.

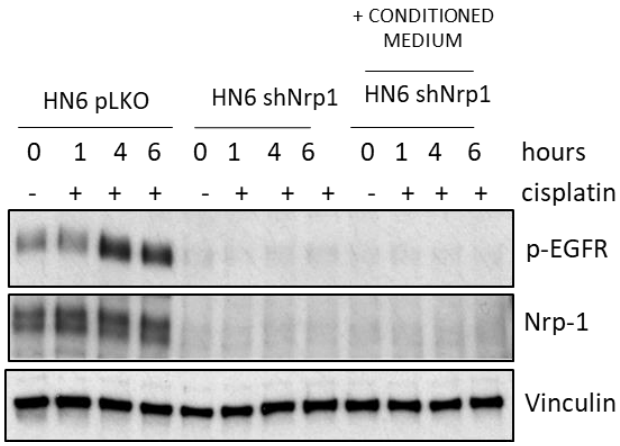


Figure 23. Time course of CDDP-EGFR activation in HN6 shNrp-1 cells, compared to HN6 shNrp-1 cells in presence of the CM produced by HN6 pLKO cells, upon cisplatin treatment, assessed by Western Blot assay.

4.8 Nrp-1 togheter with Src modulates CDDP-induced EGFR activation

The activation of EGFR in response to CDDP occurs in a Src-dependent manner (40). To examine whether the Src family kinases mediated the EGFR activation upon CDDP treatment in HN6 cells, we treated the cells with a Src family kinase inhibitor (Saracatinib) (4 μM), in combination with CDDP treatment. We observed that the Src inhibition impaired the CDDP-induced EGFR phosphorylation compared to the EGFR activation upon the CDDP treatment alone, in the HN6 plkO. This effect was absent in the Nrp-1 silenced cells, in which no EGFR phosphorylation was observed and the signaling cascade that activate AKT and MAPK was tuned off. These data showed that Nrp-1 might have a key role in association with Src kinase in modulating CDDP-induced EGFR activation in HNSCC cells.

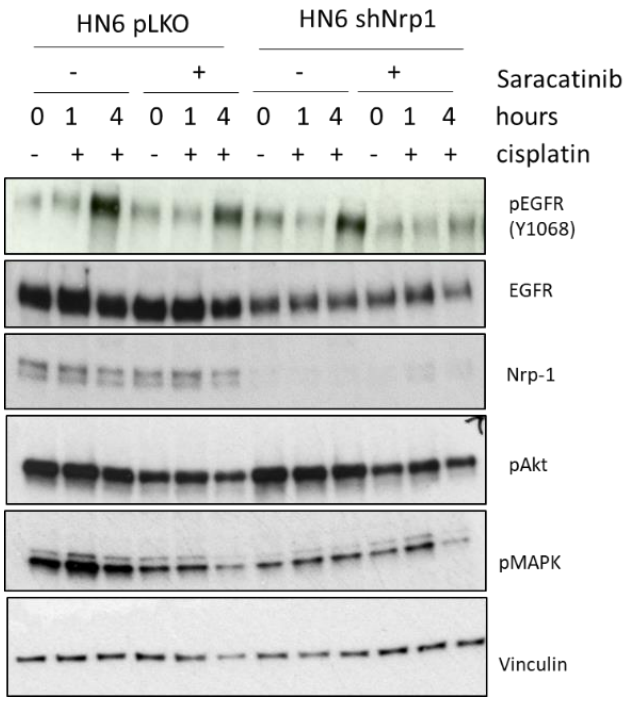


Figure 24. Time course of EGFR, AKT and MAPK activation of HN6 shNrp-1 cells compared to HN6 plkO cells, upon cisplatin treatment, in presence and absence of Saracatinib, assessed by Western Blot analysis.

CHAPTER V

-DISCUSSION

and

CONLUSIONS-

5. Discussion and conclusions

Despite great advances in the cancer research, the biology and the outcome of head and neck cancer have not been substantially changed. Up to now, the prognosis for HNSCC patients still remains determined by the disease stage at presentation. Tumor size, the presence of lymph-node metastases, distant metastases, as well as persistent infection with high risk HPVs, determine the stage (13). With respect to the therapies, the standard of care for these patients consists in surgery, radiotherapy, chemotherapy and recently immunotherapy. Notwithstanding few improvements, the 5-year survival rate for HNSCC patients remains poor, with 40-50% of mortality (27). Although concurrent radio- and chemotherapy has produced important advances, in survival and organ preservation, their use is limited by toxicity. Moreover, the loss of specificity for cancer cells for radiation and chemotherapy results in the high toxicity of these therapeutic approaches. By targeting aberrant growth factor pathways, specific for malignant cells rather than all rapidly proliferating cells, molecularly targeted therapies could offer the potential to improve the outcome without increasing the toxicity. Then, the limited information available on the biology of HNSCC claim an urgent need to search for markers of prognostic value and molecular target for novel therapeutic strategies. EGFR is one the most studied candidate. It results overexpressed in about 90 % of HNSCC and the combination of its monoclonal antibody, cetuximab, with radio and chemotherapy represents the first targeted therapy approved by FDA and is the standard of care for patients with recurrent or metastatic HNSCC (31).

Nrp-1, for its role of co-receptor of the EGF receptor, resulted an interesting candidate to investigate in HNSCC. Nrp-1 is widely expressed in a variety of human tumors but it has not been explored before in HNSCC. By performing IHC staining for Nrp-1, on a tissue microarrays of 217 patient specimens we investigated whether the Nrp-1 expression level and intracellular localization correlated with the clinicopathological characteristics of the HNSCC patients analysed. We showed that high level of Nrp-1 expression significantly correlated with a shorter overall survival, in a subgroup of patients harbouring a not oropharynx tumor and are over 60 years old. We noticed that, in this subgroup of HNSCC clinical samples, tumor stage and Nrp-1 expression correlated with a poor prognosis. Then, we performed a multivariate Cox regression analysis to identify the better risk predictor. Interestingly, Nrp-1 expression resulted predictive of poor prognosis more than the tumor stage.

Tumor cell proliferation and survival, angiogenesis and metastasis-formation and tumor immune escape are mechanisms in which Nrp-1 have a reported role (78). The capability to control multiple signaling pathways in different cellular type may contribute to the pleiotropic functions of Nrp-1, supporting the hypothesis that Nrp-1 might represent a suitable target for cancer therapies. However, the Nrp-1 role in the chemotherapy sensitivity has not been investigated before. In order to explore the Nrp-1 role in cisplatin sensitivity, we stably silenced Nrp-1 in a wide series of HNSCC cell lines. Nrp-1 depletion resulted detrimental in HN and CAL27 cells, suggesting a role for Nrp-1 in the cells survival, as already reported for lung cancer cells (73). We obtained stable Nrp-1 silencing in the CAL33, HN6 and HN13 cells, that carry EGFR wild type, amplificated, mutated and amplificated, respectively, as reported and shown in the table 1. Noteworthy, the HN6 and CAL33 cells, silenced for Nrp-1, resulted more sensitive to chemotherapy, compared to the respectively cells transduced with the control lentiviral vector.

In tumors, treatment failure through the mechanisms of resistance is often associated with activation of side signaling pathways. Indeed, CDDP-induced cytotoxic stress activates several signaling pathways, that affect cell growth and survival, cell cycle, DNA repair and drug transport (79). One of the pathway is represented by the EGFR pathway (40). Considering our data, we asked whether Nrp-1 could sustain the cisplatin induced EGFR activation. Nrp-1 is able to enhance the EGFR signaling upon ligand stimulation in several cellular models, as already described (73). Noteworthy, we observed that the Nrp-1 depletion severely impaired the cisplatin-induced EGFR phosphorylation, with also decreased activation of two EGFR downstream effectors, such as AKT and MAPK, known to sustain cellular proliferation and survival. Differently from the common rapid and transient EGFR activation in response to stimulation with its physio-logical ligands, CDDP-mediated EGFR activation occurs several hours after initiation of treatment. The delayed EGFR activation, approximately 4 h after CDDP treatment is consistent with the chemotherapic drug mechanism of action, which works with the formation of DNA adduct. This observation let us to hypostasize that Nrp-1 participates in the control of EGFR activation with a mechanism different from the ligand-dependent, as previously described. Benharv et al. reported that CDDP activates EGFR in various cell type but not in head and neck (40). They propose that EGFR activation in response to genotoxic stress is a cell survival response, since inhibition of EGFR activation enhances CDDP-induced death. Mechanistically, the authors showed that CDDP induces EGFR phosphorylation in ligand independent manner and by the mediation of the Src kinase. To shed light on the way by which Nrp-1 contribute to CDDP-

dependent EGFR activation we performed a conditioned medium experiment providing evidence that the EGFR phosphorylation observed in our cellular model is ligand-independent. In this framework, we furthermore showed that Nrp-1 works as an additional player in the regulation of EGFR signaling, together with Src, since Nrp-1 silencing combined to the Src inhibitor, Saracatinib, turned off completely EGFR activation. Few clinical trials including Saracatinib in HNSCC are ongoing (80) (81). In a translational perspective, Saracatinib could be tested in combined therapeutic regimens with Nrp-1 interfering molecules, such as nanobodies or small molecules interacting with the extracellular domain.

In conclusion, these data suggested Nrp-1 as prognostic marker in a specific subgroup of HNSCC patients, over 60 years old and characterized by not oropharynx tumor. Furthermore, we provided observations of Nrp-1 contribution to cisplatin sensitivity *in vitro*. Additionally, we expanded the repertoire of signaling in which Nrp1 is involved, showing for the first time the Nrp-1 control of cisplatin-induced EGFR signalling. This observation opens to new investigations, in order to understand the functional impact of Nrp-1 control of EGFR pathway activated in response to chemotherapy and whether Nrp-1 could be a suitable target for HNSCC therapy.

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