UNIVERSITY OF PIEMONTE ORIENTALE "AMEDEO AVOGADRO"

PhD in Molecular Medicine (Profile C):

Investigation of the role of NEU3 in colorectal carcinogenesis and in the prediction of efficacy of EGFR targeted therapies.

PhD annual report 2011/2012

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1. INTRODUCTION

1.1 Colorectal cancer: epidemiology and molecular carcinogenesis

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western countries and first when smoking-related cancers are excluded (Boyle et al, 1985; Day et al, 2003). Estimated 5-year survival rates range from more than 90% for patients with stage I disease to less than 10% for patients with metastatic CRC (Venook, 2005a). At a molecular level, intensive screening for genetic alteration led to the identification of two major types of CRC, one is characterized by normal caryotype, normal DNA index (Houlston et al, 2001) and genetic instability at microsatellite loci and is called MSI-positive cancer (Ilyas et al, 1999) and the other is characterized by alteration in APC, KRAS, TP53 and in the tumor suppressor genes at chromosome 18q (Laurent-Puig et al, 1999). In addition, several other markers have been found to be altered in CRC, and a good estimation is that more than 10-20 alterations occur in a single CRC. Current strategies in the management of CRC are focused in the identification of drugs specifically addressing these alterations and, if possible, in predicting the efficacy of these compounds (named "targeted therapies")

Therapeutic options for treating advanced CRC include the use of fluoropyrimidines, irinotecan, oxaliplatin, and targeted biological therapies (Meyerhardt et al, 2005), shifting from monotherapy to combination therapy and, more recently, to sequential combination therapy. Because these more efficacious treatment regimens allow patients to survive longer and receive more lines of therapy, choosing the best treatment regimen is becoming increasingly complex (Venook, 2005b). In particular, the combination fluouracil-oxaliplatin (FOLFOX) and fluorouracil-irinotecan (FOLFIRI) appear to be the most effective in terms of efficacy and tolerability. However, following both treatments, the overall survival is less than two years.

Chemotherapies, however, are limited by their lack of specificity and by frequent and potentially severe dose-limiting toxicities. Therefore, there is an urgent need for more effective, better-tolerated treatments that specifically target the process pivotal to tumorigenesis and metastasis. Further advances in the understanding of molecular biology have led to the development of targeted-specific agents. In the treatment of metastatic disease cetuximab and panitumumab, targeted therapies against the Epidermal Growth Factor Receptor (EGFR), have been recently introduced.

The EGFR gene, located on chromosome 7, encodes for a transmembrane receptor with intrinsic tyrosine kinase activity belonging to the HER family. Ligand binding induces receptor dimerization and autophosphorylation on several tyrosine residues in the intracellular domain, creating a series of high-affinity binding sites for various transducing molecules that are involved in transmitting the mitogenic signalling through the Ras/mitogen activated protein (MAP) kinase pathway or to the phosphatidylinositide-3-phosphate kinase (PI3K)/AKT pathway. AKT and MAP kinases in turn transduce the mitogenic signalling into the nucleus by regulating several transcription factors which control the expression of genes relevant for cell proliferation and survival (Woodburn 1999; Talapatra et al, 2004; Venook, 2005b). EGFR is involved in cell proliferation, differentiation, metastasis, angiogenesis, and programmed cell-death (Carpenter et al, 1990). It is overexpressed in a large proportion of carcinomas and is associated with disease progression and poor prognosis in CRC (Resnick et al, 2004). Cetuximab and panitumumab are two monoclonal antibodies (MoAbs) that, by their binding to the extracellular domain of EGFR, are able to block its dimerization and, therefore, its activation followed by the transduction cascade of mitogen signals. The ability of cetuximab or panitumumab for blocking the EGFR pathway is supported by preclinical and clinical studies. At preclinical level several studies suggested that

EGFR overexpression is required for cetuximab activity (Fan et al, 1993; Ciardiello et al, 1999; Baselga et al, 2000; Prewett et al, 2003). At clinical level, two phase II trials demonstrated that patients with advanced CRC had a response rate of 11% when cetuximab was administered as single agent therapy, and 23% when combined with irinotecan (Saltz et al, 2004; Cunningham et al, 2004), in irinotecan-resistant patients. As a consequence of these clinical studies, cetuximab is currently indicated for the treatment of patients with irinotecan-resistant mCRC (Venook 2005a). These data suggest that cetuximab is effective in a subgroup of patients with mCRC. Importantly, these two studies included only patients with immunohistochemical evidence of EGFR expression. However, about this methodology of EGFR evaluation, there are several problems in interpreting EGFR deregulation. Indeed, EGFR analysis by immunohistochemistry does not seem to represent the best way to evaluate EGFR alterations, since it has been shown that the type of fixative used, the storage time of unstained tissue sections (Atkins et al, 2004), the type of primary antibody used (Kersting, et al, 2006) and the methods of immunohistochemistry analyses and/or evaluation (Langner et al, 2004) might generate conflicting data. Moreover, it has also been demonstrated that EGFR-negative patients, as determined by immunohistochemistry, may respond to cetuximab-based therapies (Chung et al, 2005).

On the contrary, it appeared that EGFR gene copy number evaluated by fluorescent in situ hybridization (FISH) might better predict cetuximab response in advanced CRC. At first, one study (Moroni et al, 2005) showed that eight out of nine patients with objective response to the drug had an increased EGFR copy number (named copy number gain, or CNG), due to either EGFR gene amplification or polysomy of chromosome 17. These results were confirmed in CRC cell lines treated with cetuximab and by other clinical studies in small cohort of patients, including one of our group on patients resected and treated in Ticino (Lièvre et al, 2006; Frattini et al, 2007; Sartore-Bianchi et al, 2007; Cappuzzo et al, 2008). Overall, these results pointed out that patients with EGFR gene amplification and with at least three copies/nucleus of EGFR gene may benefit from anti-EGFR MoAbs, whereas patients with a disomic pattern are more likely to be refractory to these targeted therapies.

After dimerization and cross-autophosphorylation, EGFR transduces the mitogenic signalling through two main pathways: 1) the MAP kinase pathway, that includes the sequential activation of KRAS, BRAF, MEK and ERK, mainly involved in cell proliferation, and 2) the PI3K-PTEN-AKT pathway, that leads to the activation of mTOR and that is mainly involved in cell survival (Woodburn 1999; Talapatra et al, 2001; Venook, 2005b).

A body of evidence arising from several reports published in the last three years (including our research group) has shown that the presence of KRAS mutations leads to resistance to EGFR-targeted therapies in mCRC (Siena et al, 2010). Based on these results, the two international agencies FDA and EMA have approved the use of cetuximab and panitumumab only for mCRC patients whose tumors display a KRAS wild-type sequence.

Starting from this platform, several research groups, including our, have investigated in a retrospective manner whether alterations occurring in other markers belonging to the EGFR cascade could be predictive of the efficacy of these drugs. The same effect of KRAS mutations has been proposed for BRAF and for PIK3CA mutations, and for PTEN loss of expression. However, at the moment, the negative predictive role played by these alterations has not been fully determined because, although the results obtained by different groups are not contradictory and confirmed earlier results, they come from too few retrospective studies. In addition, it has also been proposed that if a combined analysis of KRAS, BRAF, PIK3CA and PTEN is performed in the same cohort, it is possible to identify up to 70% of patients who cannot benefit from EGFR-targeted therapies in colorectal cancer (reviewed in Siena et al, 2010).

Overall, at the moment, only KRAS mutational testing has entered in clinical practice.

1.2 EGFR ligands: Amphiregulin and Epiregulin

Amphiregulin (AREG) and Epiregulin (EREG) belong to the epidermal growth factor (EGF) family, and act as mitogenic stimulators through binding to EGFRs (Inatomi O et al, 2006)
AREG is produced as a transmembrane precursor that is released from the cell membrane by a disintegrin and metalloproteinases (ADAM17) (Lu XQ et al, 2009). Elevated levels of AREG have been characterized in various types of tumors, and amphiregulin is assumed to play a role in promoting cancer progression (Yamada M et al, 2008; Gilmore JL et al, 2009).
AREG has been implicated in the growth and regeneration of intestinal mucosa and might be related to the development and progression of gastrointestinal tumors (Berasain C et al, 2005; Michalopoulos GK et al, 2005).
AREG also elevates angiogenic activity, promoting tumour growth and metastasis in pancreatic, colorectal, liver, and lung cancers (Yamada M et al, 2008). Patients with high levels of AREG display poorer prognosis and higher resistance to cetuximab than patients with low levels of AREG (Berasain C et al, 2005; Addison CL et al, 2010; Yotsumoto F et al, 2010).
EREG is a novel member of the EGF family initially purified from conditioned medium of the mouse fibroblast-derived tumor cell line NIH-3T3 clone T7 (Toyoda H et al, 1995). The coding sequence of human EREG cDNA predicts a 46-amino acid single-chain polypeptide, exhibiting 24–50% homology with the sequences of other EGF receptor (EGFR)-ligands. It binds to EGFR and is a potent mitogen for rat primary hepatocytes. EREG exhibits a bifunctional regulatory property in that it inhibits the growth of several epithelial cell lines and stimulates the growth of fibroblasts and various other cell types (Toyoda et al, 1995; Toyoda H et al, 1997).

1.3 Glycosylation - sialidases

Asparagine (N)-linked glycosylation is a highly regulated process that produces a large and diverse repertoire of cellular glycans that are mostly attached to proteins (Schwarz et al, 2011). Abnormal glycosylation is known to be associated with cancer malignancy (Ohtsubo et al, 2006). Among the sugars found on the cell surface there are sialic acids, which exist as terminal monosaccharide attached to cell surface glycan chains. The variety of sialic acid decorations on the cell surface governs many biological processes, including cell recognition, cell adhesion, receptor activation, and signal transduction (Varki et al, 2007). Studies performed over the last decade have focused on the involvement of sialylation in the progression of cancer (Ohtsubo et al, 2006; Varki et al, 2007), but the actual function of sialylation in tumorigenesis has received much less research attention (Schwarz et al, 2011). Recently, the EGFR was identified as one of the sialylated glycoproteins in human lung cancer (Liu et al, 2011). In fact, it has been shown that sialylation is capable of regulating EGFR activity (Liu et al, 2011). Thus, understanding the regulation of EGFR glycosylation may provide novel insights into cancer biology and suggest possible therapeutic strategies. Recently, it has been demonstrated that the sialyltransferase ST6Gal-I induces adhesion and migration, and promotes radioresistance and protection from apoptosis in colon cancer cells (Lee et al, 2008; Lee et al, 2010a; Lee et al, 2010b) and that ST6Gal-I overexpression significantly affects EGF-mediated cell growth and induces chemoresistance to gefitinib (a tyrosine kinase inhibitor against EGFR) in colon cancer cells (Park et al, 2012). These data therefore point out that the absence of sialic acid could play a relevant role in the activation of EGFR and, as a consequence, in the efficacy of EGFR-targeted therapies.
However, the main family of enzymes able to regulate the level of sialic acid is represented by sialidases, that can directly activate EGFR (Soderquist et al, 1984).

1.4 Human sialidase NEU3

Four different sialidases have been described in mammals: lysosomal (NEU1), cytosolic (NEU2), plasma-membrane (NEU3), and mitochondrial/lysosomal/intracellular membrane (NEU4). These enzymes differ in their subcellular localizations, pH optima, kinetic properties, responses to ions and detergents, and substrate specificities. There appears to be little overlap in function of the individual sialidases, despite their shared mechanism of action.
The membrane-associated sialidase NEU3 has been so far the most studied enzyme of the family. First cloned from bovine brain in 1999 as a plasma membrane associated sialidase specific for gangliosides (Miyagi et al, 1999), NEU3 was subsequently characterized at molecular level from various mammalian species, thus confirming in *in vitro* assays its high substrate specificity for gangliosides (Monti et al, 2002). NEU3 plays a relevant role in several cellular processes such as cell differentiation, proliferation and apoptosis. Indeed the constitutive silencing of the enzyme with siRNA in murine C2C12 myoblasts induced (i) complete inhibition of the differentiation processes; (ii) massive apoptosis upon differentiation conditions or in confluence cell cultures; (iii) EGFR inhibition and down-regulation as a consequence of the increased levels of endogenous ganglioside GM3 (Jacquel et al, 2006).

1.5 Sialidase NEU3 and cancer

In the last few years many scientific papers have been published concerning the involvement of sialidases, in particular NEU3, in various kind of tumors (Miyagi et al, 2004; Miyagi, 2008; Miyagi et al, 2008 a, b, c). Human NEU3 was found to be up-regulated in human colon cancer (Kakugawa et al, 2002) and in vitro experiments demonstrated that it is also involved in the regulation of cell proliferation through integrin mediated signaling (Miyagi, 2008). Recently, the effect of NEU3 in promoting tumorigenesis in vivo has been reported (Shiozaki et al, 2008). NEU3 mRNA levels have also been found to be significantly increased in renal cell carcinomas and in prostate cancer, where there is a correlation with malignancy as assessed by the Gleason score (Ueno et al, 2006; Kawamura et al, 2011). In prostate cancer cell lines, forced overexpression of NEU3 leads to a significant induced androgen receptor, and this effect was abrogated by inhibitors of PI3K and MAPK pathways (Kawamura et al, 2011). In addition, NEU3 interacts directly with signaling molecules such as EGFR as demonstrated by co-immunoprecipitation experiments. In cancer cells NEU3 suppresses apoptosis increasing the receptor phosphorylation and thus the activation of the Ras/ERK pathway (Wada et al, 2007). In general, it has been demonstrated that NEU3 suppresses apoptosis of cancer cells by promoting EGFR phosphorylation, and consequent activation of EGFR downstream pathways, mainly through the MAPK pathway.

2. AIM

The aim of this doctorate was to investigate the role of NEU3, a sialidase enzyme that interacts with EGFR. The role of NEU3 in colorectal carcinogenesis is largely unknown. We proposed to evaluate the correlation of NEU3 with markers belonging to the EGFR pathways and with those mainly involved in colorectal carcinogenesis. In addition, by modulating the levels of NEU3 expression in cellular models, and by relating these data to the administration of monoclonal antibodies against EGFR, we think we will be able to significantly increase the knowledge on EGFR-targeted therapies efficacy in colorectal cancer (CRC) patients.

The project was scheduled as follows:

AIM1: analysis of NEU3 deregulation with respect to alterations occurring in EGFR pathways and in particular:

1. correlation between EGFR and NEU3 deregulation
2. correlation of NEU3 expression with alterations occurring in EGFR downstream pathways

AIM2: Association of NEU3 expression with the gene alterations involved in the classical model of colorectal cancer development.
AIM3: investigation of the role of NEU3 expression in the prediction of efficacy of EGFR-targeted therapies.

In the first year we achieved AIM1.

3. MATERIALS AND METHODS

3.1 EGFR and NEU3 deregulation

We investigated 73 patients surgically resected for a CRC. Fresh tissues from both primary tumors and paired normal mucosa were immediately freezed in liquid nitrogen and subsequently stored at -80°C until the analysis.
Total RNA was isolated from primary tumors or from adjacent normal mucosa by the RNeasy kit (QIAGEN) as recommended by the manufacturer. First strand cDNAs were synthesized by reverse transcription (Superscript II, Invitrogen, Life Technology, Carlsbad, California USA) and used as templates for real-time PCR experiments (SYBR-green assay) that were performed on a CFX96 Real-time PCR system (Bio-Rad, Hercules, California USA). In this cohort of patients we found that NEU3 and EGFR mRNA levels vary markedly from patient to patient both in normal mucosa and in tumors. As a reference gene, we decided to use pol2 since it showed high stability in the analyses. The fold increase in tumor was calculated through the $2^{-\Delta\Delta Ct}$ method using paired normal tissue as calibrator. We considered gene overexpressing tumors those showing ≥ 3-fold expression level with respect to paired normal mucosa.

3.2 Fluorescence in situ Hybridisation (FISH)

FISH was performed on 3-μm formalin-fixed, paraffin-embedded tissue sections treated using the Paraffin Pretreatment kit II (Pretreatment Reagent VP 2000, Abbott Molecular AG, Baar, Switzerland) according to the manufacturer's instructions. The dual color EGFR FISH assay was perfomed using LSI EGFR/CEP7 probes (Vysis, Downer's Grove, IL, USA) as mentioned earlier (Martin et al, 2009). The signals were evaluated with a fluorescent automated microscope (Zeiss Axioplan 2 Imaging, Oberkochen, Germany) equipped with single and triple band pass filters. Images for documentation were captured using an AxioCam camera (Zeiss AxioCam MRm) and processed using the AxioVysion Software (Zeiss). Patients were classified using descriptive criteria: cases showing 2 chromosomes 7 in more than 60% of cells were defined as disomic; patients with 3 or 4 chromosomes 7 in >40% of cells were defined as low polisomic; tumour samples with an aberrant number of chromosome 7, defined as >4 copies in >40% of cells, were classified as high polysomic; specimens with a ratio >2 between EGFR gene and chromosome 7 centromere signals in >10% of cells were defined as carrying EGFR gene amplification. According to the literature, patients carrying either a high polysomic profile or gene amplification were classified as FISH+; patients carrying either a low polysomic profile or disomic profile were classified as FISH-.

3.3 Mutational status of *KRAS*, *BRAF* and *PIK3CA*

Genomic DNA was extracted from 3-μm formalin-fixed, paraffin-embedded tissue sections using QIAamp Mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. We searched for point mutations in *KRAS* exon 2 (including codons 12 and 13) and exon 3 (including codon 61), as already reported (Frattini et al, 2007). We investigated *BRAF* point mutations in exon 15 (including codon 600) and *PIK3CA* point mutations in exons 9 (including codons 542 and 545) and 20 (codon 1047) as previously described (Moroni et al, 2005; Frattini et al, 2007) because in these regions more than 95% of activating mutations occur in each gene. Sequencing of the PCR products was done using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analysed with appropriate software (SeqScape Software Version 2.5,

Applied Biosystems). Each sequence reaction was performed at least twice, starting from independent PCR reactions.

3.4 AREG and EREG expression analysis

AREG and EREG expression was investigated by Real-time PCR using a Taq-Man assay, as reported in the literature (Baker et al, 2011), on a CFX96 Real-time PCR system (Bio-Rad). As a reference gene, we decided to use pol2 since it showed high stability in the analyses. The fold increase in tumor was calculated through the $2^{-\Delta\Delta Ct}$ method using paired normal tissue as calibrator. We considered gene overexpressing tumors those showing ≥ 3-fold expression level with respect to paired normal mucosa.

4. RESULTS

4.1 NEU3

As shown in Figure 1, if we consider that a tumor can be classified as NEU3 overexpressing when its level is more than 3-fold with respect to paired normal mucosa, 30 out of 73 cases (41%) showed NEU3 mRNA overexpression. Two cases (#49 and #56) are borderline.

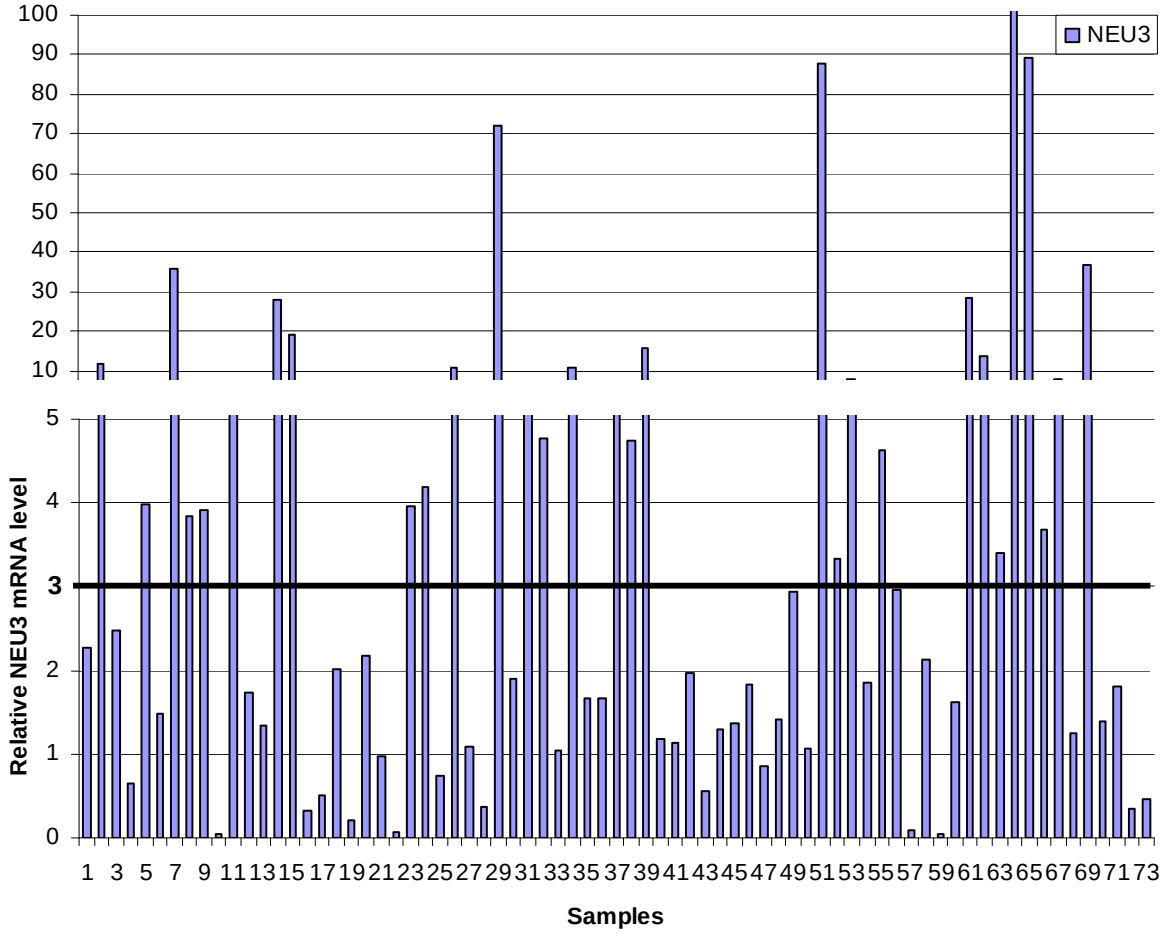

Figure 1: NEU3 expression in 73 patients. Real-time PCR analysis of NEU3 levels in tumor tissue of 73 patients. mRNA expression levels were normalized to pol2 mRNA and to paired normal mucosa. A gene can be considered overexpressed when its fold is above 3 (black line). The experiments were performed in triplicate.

4.2 EGFR

As shown in Figure 2, if we consider that a tumor can be classified as EGFR overexpressing when its level is more than 3-fold with respect to paired normal mucosa, 14 out of 73 cases (19.2%) showed EGFR mRNA overexpression.

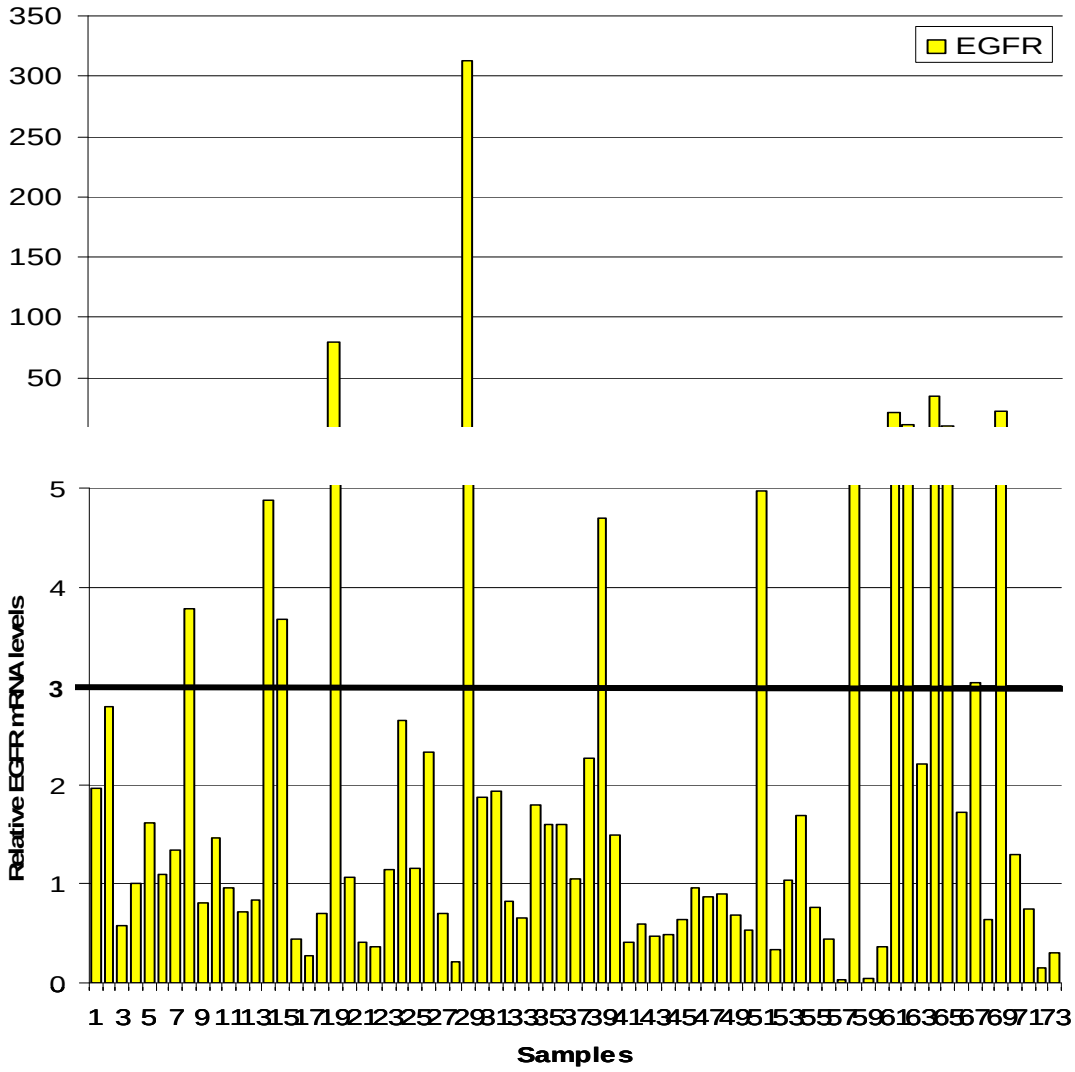

Figure 2: EGFR expression in 73 patients. Real-time PCR analysis of EGFR levels in tumor tissue of 73 patients. mRNA expression levels were normalized to pol2 mRNA and to paired normal mucosa. A gene can be considered overexpressed when its fold is above 3 (black line). The experiments were performed in triplicate.

4.3 Correlation between NEU3 and EGFR expression

By comparing NEU3 and EGFR mRNA expression levels, we observed that NEU3 was overexpressed in 18 out of 59 (30.5%) EGFR negative cases and in 12 out of 14 (85.7%) EGFR overexpressing cases. This difference is statistically significant ($p=0.0002$, two-tailed Fisher's Exact Test). These data suggest therefore that a strict correlation between NEU3 and EGFR mRNA expression exists (Figure 3).

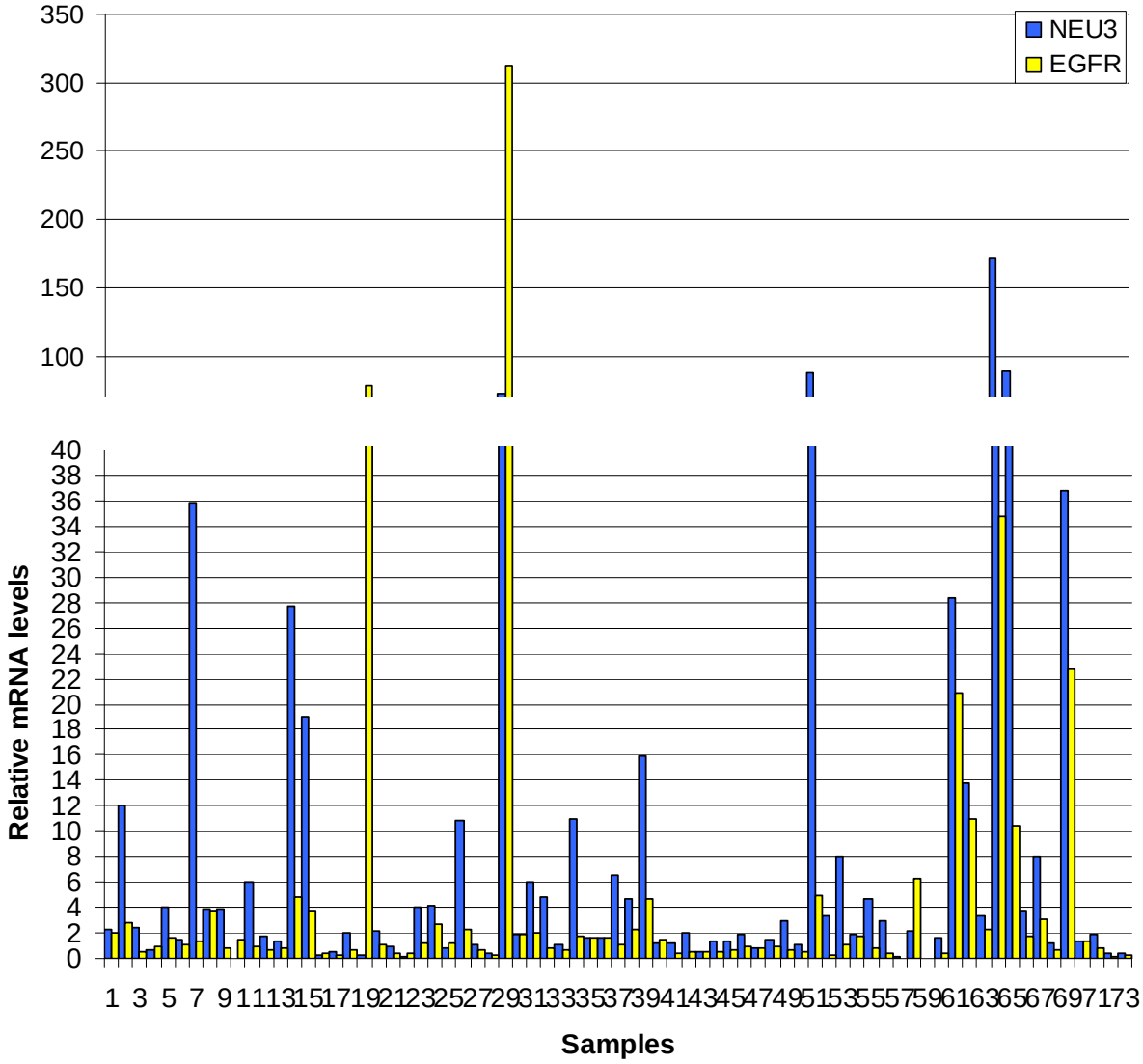

Figure 3: NEU3 and EGFR expression in 73 patients. Real-time PCR analysis of NEU3 and EGFR mRNA levels in tumor tissue of 73 patients. mRNA expression levels were normalized to pol2 mRNA and to respective normal mucosa. A gene can be considered overexpressed when its fold is above 3. The experiments were performed in triplicate.

4.4 EGFR gene status by FISH

To date, we analyzed 19 cases and we found that 3 patients were not evaluable, 8 patients were classified as FISH+ and 8 patients were classified as FISH-. Within FISH+ patients we observed: 2 patients with high level of amplification (R=3 and R=4, respectively), 1 patient with low level of amplification (R<3), 4 patients with high polisomy (HP) and 1 patient with low polisomy (LP, tetrasomy in >40% of cell population). Within FISH- patients we observed: 4 patients with disomy (2n), 1 patient with low polisomy (LP, trisomy and/or tetrasomy in <40% of cell population) and 3 patients with concomitant disomy and low polisomy (2n+LP). FISH analysis is now on-going on the remaining cases of our cohort of patients. Subsequent correlation analyses by Fisher's Exact

Test to evaluate the possible correlations existing between EGFR gene status by FISH and EGFR mRNA levels by real-time PCR will be performed on the entire cohort of patients during the next year.

4.5 Correlation of NEU3 expression with alterations occurring in EGFR downstream pathways.

To date, mutational results are completed for 67 patients for KRAS gene, 68 patients for BRAF gene and 16 patients for PIK3CA gene by direct sequencing. We identified KRAS mutations in 26 samples (38.8%), 24 in codon 12 and 2 in codon 13 (Table 1). The most diffused mutations identified were the G12V (detected in 9 patients) and the G12D (in 7 cases); the other mutation observed were: G12C in 3 cases, G12A and G12S in 2 cases respectively, G12R in 1 case. All mutations occurring at codon 13 were represented by the classical change G13D (Table 1). We found only 1 mutated case (1.5%) for BRAF gene; the mutation was the classical change V600E (Table 1). We observed 1 mutation (6.25%) out of 16 evaluable patients for PIK3CA gene; the mutation was the E542K change in codon 9 (Table 1). The frequency and the type of mutations perfectly match with those reported in the literature.
Using the two-tailed Fisher's Exact Test, we evaluated possible correlations existing between these molecular markers (KRAS, BRAF, PIK3CA) and NEU3 and EGFR mRNA expression levels. No correlation was observed among markers, probably due to the small number of patients analyzed until now.

Sample	KRAS	BRAF	PIK3CA
#1	WT	V600E	WT
#2	G13D	WT	WT
#3	WT	WT	WT
# 4	WT	WT	WT
# 5	WT	WT	WT
# 6	G12V	WT	WT
#7	WT	WT	WT
#8	G12V	WT	WT
#9	G12V	WT	WT
#10	WT	WT	WT
#11	WT	WT	WT
#12	G12D	WT	-
#13	WT	WT	-
#14	G12C	WT	WT
#15	WT	WT	WT
#16	G12D	WT	WT
#17	G12D	WT	E542K
#18	WT	WT	WT
#19	WT	WT	WT
#20	WT	WT	-
#21	WT	WT	-
#22	G12C	WT	-
#23	WT	WT	-
#24	G12C	WT	-
#25	G12D	WT	-
#26	WT	WT	-
#27	WT	WT	-
#28	WT	WT	-
#29	G12D	WT	-
#30	G12V	WT	-
#31	WT	WT	-
#32	WT	WT	-
#33	WT	WT	-
#34	G12A	WT	-
#35	WT	WT	-
#36	G12V	WT	-
#37	G13D	WT	-

#38	G12V	WT	-
#39	G12R	WT	-
#40	WT	WT	-
#41	WT	WT	-
#42	WT	WT	-
#43	WT	WT	-
#44	G12A	WT	-
#45	WT	WT	-
#46	WT	WT	-
#47	WT	WT	-
#48	G12V	WT	-
#49	G12V	WT	-
#50	WT	WT	-
#51	-	WT	-
#52	G12S	WT	-
#53	WT	WT	-
#54	WT	WT	-
#55	WT	WT	-
#56	G12S	WT	-
#57	G12D	WT	-
#58	WT	WT	-
#59	G12D	WT	-
#60	WT	WT	-
#61	G12V	WT	-
#62	WT	WT	-
#63	WT	WT	-
#64	WT	WT	-
#65	WT	WT	-
#66	WT	WT	-
#67	WT	WT	-
#68	WT	WT	-

Table 1: Results reporting the KRAS, BRAF and PI3KCA mutations.

4.6 AREG and EREG

We observed AREG overexpression in 33 out of 57 (57.9%) analyzed cases (Figure 4) and EREG overexpression in 34 out of 53 (64%) analyzed cases (Figure 5). At first we correlated the expression of the two ligands. We observed that AREG overexpression was detected in 3 out of 19

(15.8%) EREG negative cases and in 27 out of 34 (79.4%) EREG overexpressing cases. This result is statistically significant ($p<0.001$, two-tailed Fisher's Exact Test) (Figure 6). No other correlation was observed among AREG and EREG and EGFR or NEU3, probably due to the small number of patients of our cohort. Correlation analyses will be done on the entire cohort of patients during the next year.

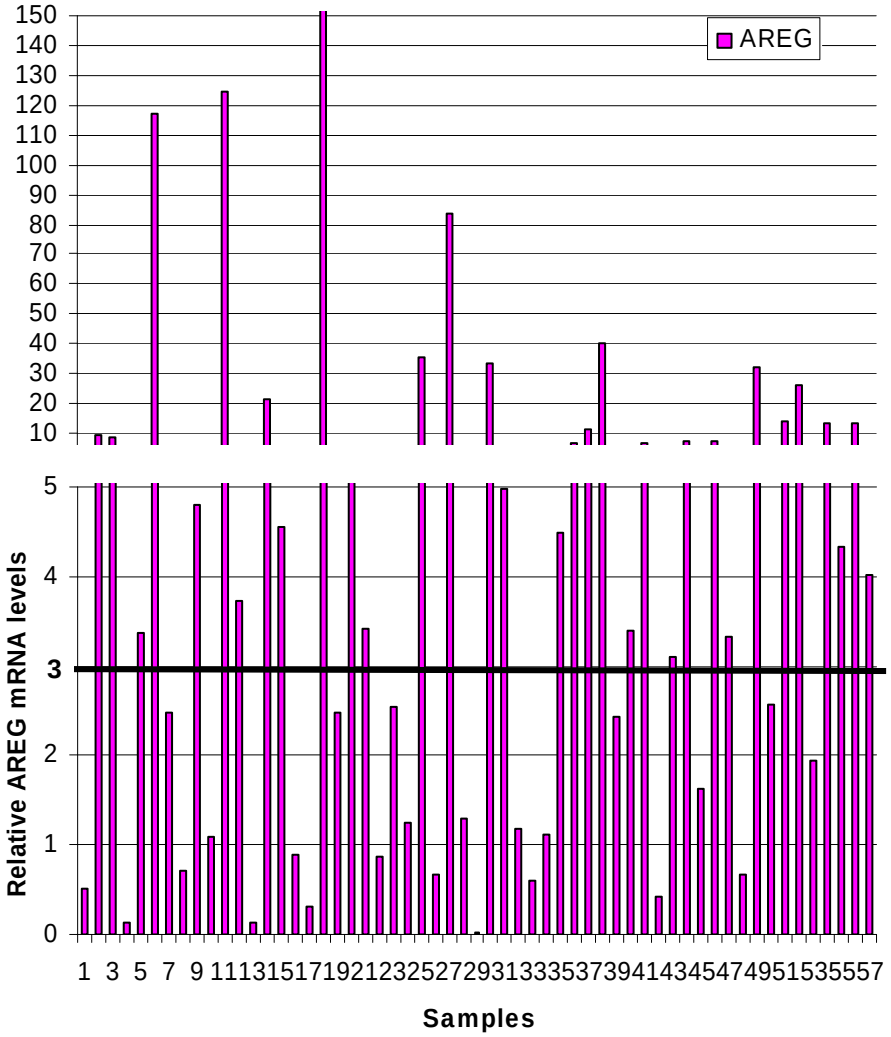

Figure 4: AREG expression in 57 patients. Real-time PCR analysis of AREG mRNA levels in tumor tissue of 57 patients. mRNA expression levels were normalized to pol2 mRNA and to respective normal mucosa. A gene can be considered overexpressed when its fold is above 3 (black line). The experiments were performed in triplicate.

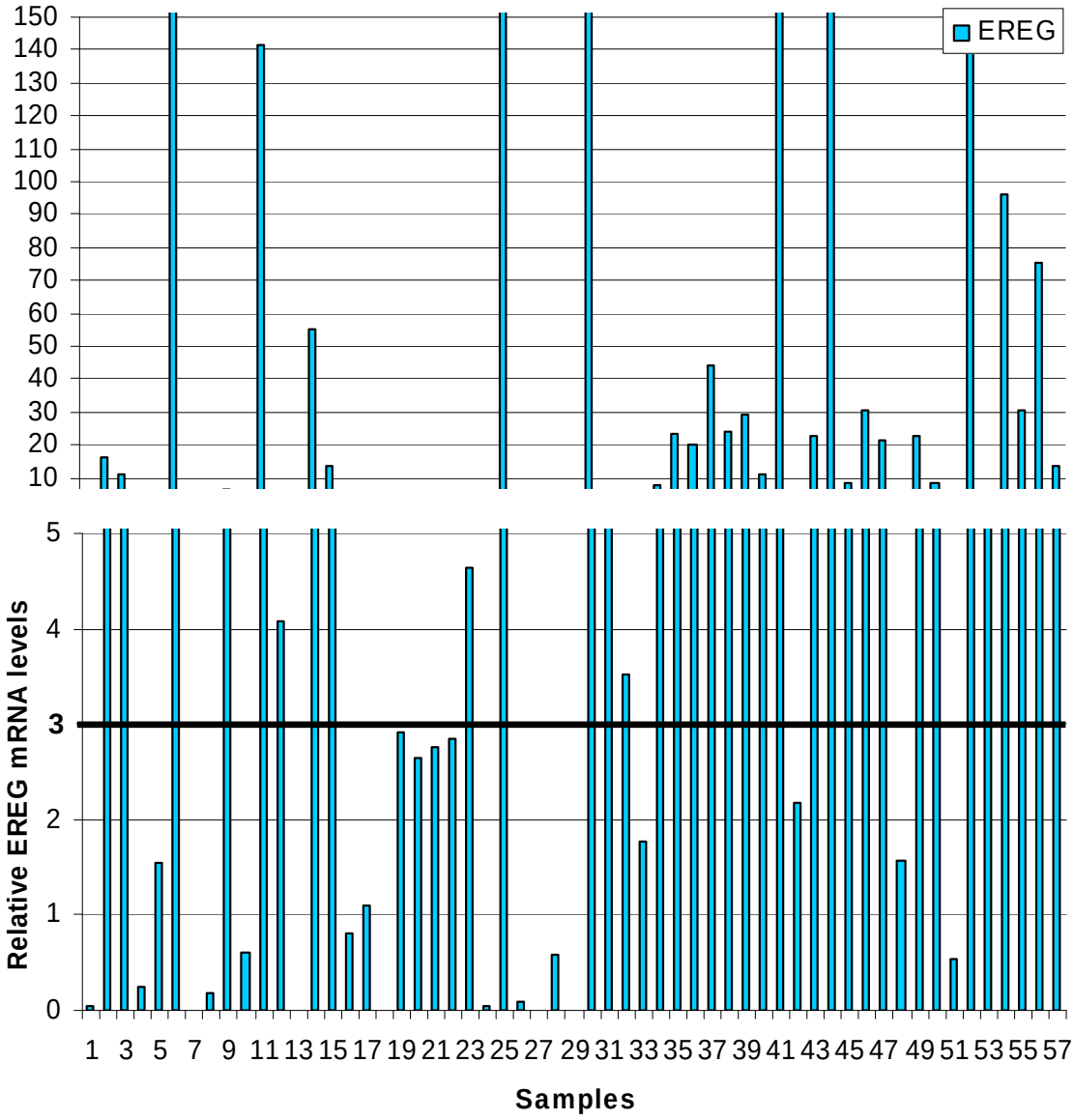

Figure 5: EREG expression in 57 patients. Real-time PCR analysis of EREG mRNA levels in tumor tissue of 57 patients. mRNA expression levels were normalized to pol2 mRNA and to respective normal mucosa. A gene can be considered overexpressed when its fold is above 3 (black line). The experiments were performed in triplicate.

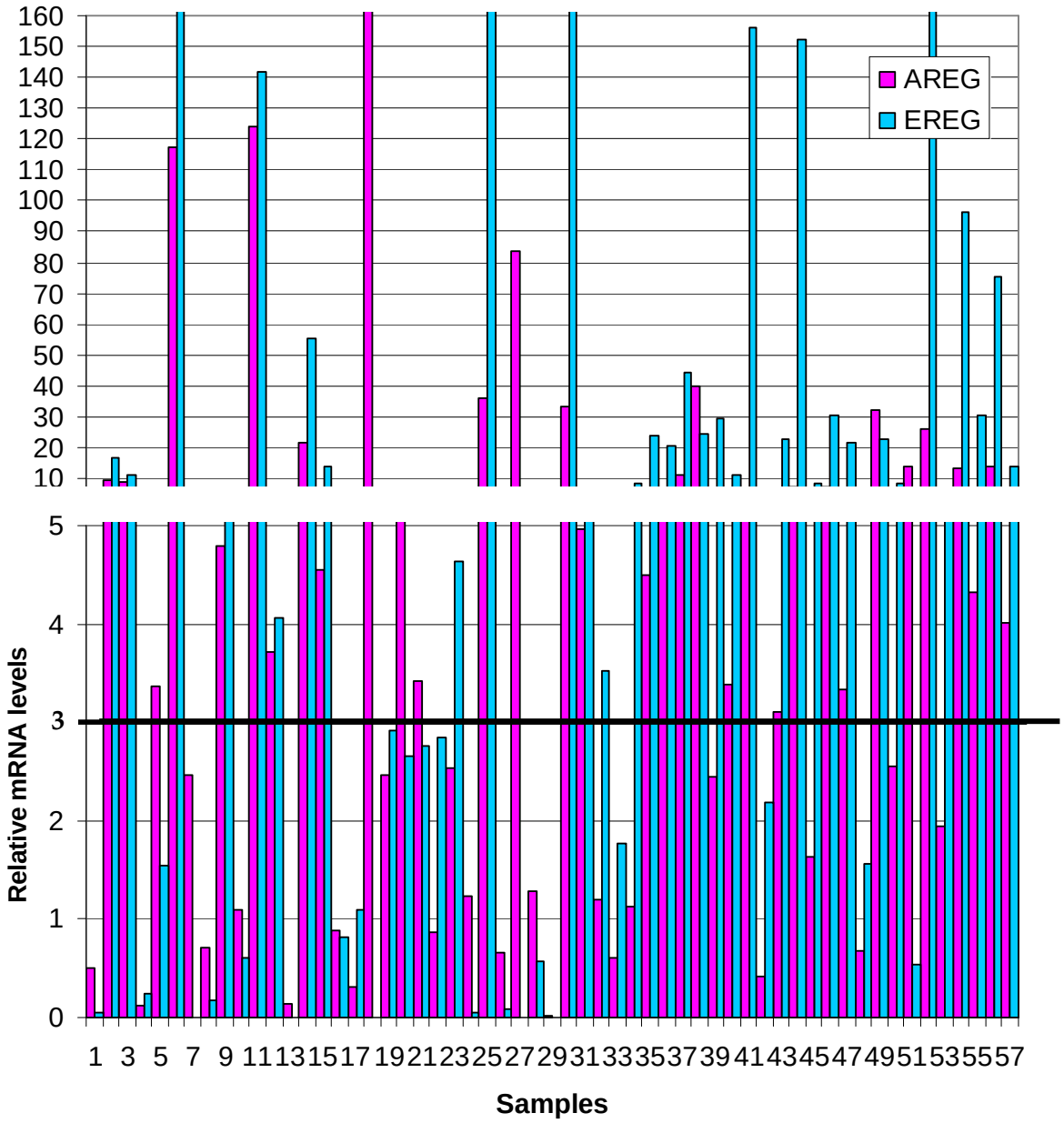

Figure 6: AREG and EREG expression in 57 patients. Real-time PCR analysis of AREG and EREG mRNA levels in tumor tissue of 57 patients. mRNA expression levels were normalized to pol2 mRNA and to respective normal mucosa. A gene can be considered overexpressed when its fold is above 3 (black line). The experiments were performed in triplicate.

5. DISCUSSION

We characterized the NEU3 and EGFR mRNA expression in 73 pathological specimens from patients affected by CRC. As it has been demonstrated that NEU3 protein co-immunoprecipitates with EGFR, we tested whether it exists a possible correlation between NEU3 and EGFR mRNA expression levels and we found that a strict correlation exists.

There is just one work in the literature where the NEU3 expression at mRNA level was investigated. Miyagi and colleagues showed in a Japanese cohort of CRC patients that NEU3 expression was increased by 3 to 100-fold in all cases (Miyagi et al, 2008). On the contrary, we found that only 41% of our cohort (including Western population only) showed NEU3 overexpression. In addition, few patients of our series displayed higher levels of NEU3 than those observed in the Japanese cohort. These discrepancies can be explained by ethnical differences, since Western and Japanese population are characterized by significant different lifestyles. On the other hand, this finding is not surprising because, for example, it has been reported that EGFR inactivation (in lung cancer is significantly different between Japanese and Western population. In Japanese patients, EGFR mutations arise at a higher rate (about 75% of cases) whereas are rarer in the Western population (10-15%) where EGFR is essentially deregulated following gene amplification.

To deepen evaluate the correlation between EGFR and NEU3 and to evaluate whether NEU3 expression can affect the activation of EGFR also at protein level, we have planned to set-up protein extraction with subsequent Western Blot experiments, for the evaluation of NEU3 and EGFR protein expression and EGFR phosphorylated protein expression.

As it has been widely demonstrated that EGFR expression can be significantly altered also through the copy number gain, we also started with the analysis of the EGFR gene status by Fluorescent in situ Hybridization (FISH). FISH analysis is completed in 19 cases and is now on-going on the remaining cases of our cohort of patients. Subsequent correlation analyses by Fisher's Exact Test to evaluate the possible correlations existing between EGFR gene status by FISH and EGFR mRNA levels by real-time PCR will be performed on the entire cohort of patients during the next year.

As KRAS, BRAF and PIK3CA may be deregulated in a consistent number of CRC and as it seems that NEU3 and EGFR expression are correlated, we proposed to investigate whether NEU3 expression levels could be related also to deregulations in EGFR downstream pathways. No correlation was observed among markers, probably due to the small number of patients analyzed until now. A more definitive answer to possible correlations will be done after the analysis of the whole cohort (next year). During first year of doctorate we started with mutations analyses occurring in these molecular markers. During next year we will end these analyses and we will set-up Western Blot experiments for the evaluation of AKT, MEK, ERK and PTEN protein expression, both in their total and phosphorylated forms.

Besides the KRAS, BRAF, PIK3CA mutational status, EGFR amphiregulin (AREG) and epiregulin (EREG) ligands' expression in CRC tumors has been shown to significantly predict clinical outcome in KRAS wild-type mCRC patients treated with anti-EGFR MoAbs cetuximab and panitumumab (Khambata-Ford et al, 2007; Loupakis et al, 2009; Saridaki et al, 2011). Our results on AREG and EREG confirm the strict correlation existing between the two EGFR ligands, as previously reported (Baker et al, 2011). No other correlation was observed among AREG and EREG and EGFR or NEU3. Correlation analyses will be done on the entire cohort of patients during next year.

Finally, following a recent paper investigating alterations of a sialyltransferase enzyme, in particular ST6Gal-1, an enzyme with an opposite effect with respect to sialidases (Park et al, 2012), we propose to evaluate whether NEU3 deregulation may revert, at least partially, the EGFR-

targeted therapies resistance due to the presence of mutations in EGFR downstream members, thus opening new perspectives for patients who cannot benefit from monoclonal antibodies against EGFR.
The generated data may provide important information for improving the prediction of tailored chemotherapeutic regimens, to avoid inefficacious treatments, and to maintain the costs related to the clinical use of these drugs under control.

6. REFERENCES

- Addison CL et al, 2010 J. Clin. Oncol. 28: 5247–5256.
- Atkins D et al, J Histochem Cytochem 2004; 52:893-901.
- Baker JB et al, BJC 2011; 104: 488 – 495.
- Baselga J et al, J Clin Oncol 2000;18:904–914.
- Berasain C et al, Gastroenterology 2005;128:424–32.
- Boyle P et al, Int J Cancer 1985; 36:9-187.
- Cappuzzo F et al, Ann Oncol 2008; 19:717-723.
- Carpenter G et al, J Biol Chem 1990; 265:7709-7712.
- Chung KY et al, J Clin Oncol 2005; 23:1803-1810.
- Ciardiello F et al, Clin Cancer Res 1999; 5:909–916.
- Cunningham D et al, Engl J Med 2004; 351:337-345.
- Day DW et al, Oxford: Blackwell Publishing, 2003.
- Fan Z et al, Cancer Res 1993;53:4637–4642.
- Frattini M et al, Br J Cancer 2007;97:1139-1145.
- Gilmore JL et al, 2009 Mol. Cancer Res. 7: 1714–1728.
- Houlston RS. J Clin Pathol Mol Pathol 2001; 54:206-214.
- Ilyas M et al, Eur J Cancer 1999;35:335-351.
- Inatomi O et al, Int J Mol 2006 ; 18(3) :497-503.
- Jacquel A et al, Oncogene 2006.
- Kakugawa Y et al, Proc Natl Acad Sci U S A 2002.
- Kawamura et al, Cell Death Diff 2011; 19:170.
- Kersting C et al, J Clin Pathol 2006; 59:585-590.
- Khambata-Ford et al, J Clin Oncol 2007; 25(22):3230-7.
- Langner C et al, J Clin Pathol 2004; 57:773-775.
- Laurent-Puig P et al, Eur J Cancer Prev 1999; 8:S39-S47.
- Lee M et al, Cancer Res 2008.
- Lee M et al, Int J Radiat Oncol Biol Phys 2010 (a).
- Lee M et al, Oncol Rep 2010 (b).
- Lièvre A et al, Cancer Res 2006; 66:3992-3995.
- Liu YC et al, Proc Natl Acad Sci USA 2011.
- Loupakis et al, J Clin Oncol; 27:2622-2629.
- Lu XQ et al 2009 Genes Dev. 23: 1882–1894.
- Meyerhardt JA et al, N Engl J Med 2005; 352:476-487.
- Martin V et al, J Clin Pathol 2009; 62:314-24.
- Michalopoulos GK et al, Gastroenterology 2005;128:503–*6*.
- Miyagi T et al, J Biol Chem (1999).
- Miyagi T et al, Glycocon J 2004.
- Miyagi T et al, Biochim Biophys Acta 2008 (a).
- Miyagi T et al, J Biochem 2008 (b).
- Miyagi T et al, Proteomics 2008 (c).
- Miyagi T, Proc Jpn Acad Ser B Phys Biol Sci 2008 (d).
- Monti E et al, Neurochem Res 2002.
- Moroni M et al, Lancet Oncol 2005; 6:279-286.
- Ohtsubo K et al, Cell 2006.
- Park JJ et al, Biochem Pharmacol 2012; 83:849-857.
- Prewett MC et al, Clin Cancer Res 2002; 8:994–1003.

- Resnick MB et al, Clin Cancer Res 2004;10:3069-3075.
- Saltz LB et al, J Clin Oncol 2004; 22:1201-1208.
- Sartore-Bianchi A et al, J Clin Oncol 2007; 25:3238-3245.
- Saridaki et al, PLoS One. 2011 Jan 20; 6(1):e15980.
- Sasaki A et al, J Biol Chem 2003.
- Schwarz F et al, Curr Opin Struct Biol 2011.
- Toyoda H. et al, 1995 J. Biol. Chem. 270:7495–7500.
- Toyoda H. et al, 1997 Biochem. J. 326:69–75.
- Yamada M et al, Clin. Cancer Res. 2008; 14: 2351–2356.
- Yotsumoto F et al, Cancer Sci. 2010; 101: 2351–2360.

FORMAZIONE

Formazione interna

Svolta presso l'Istituto Cantonale di Patologia, Locarno (Svizzera).

Anno 2011

DATA	TITOLO	RELATORE
8 Novembre	L'evoluzione epidemiologica delle neoplasie polmonari	Andrea Bordoni
29 Novembre	Trattamenti chemioterapici delle neoplasie polmonari	Patrizia Froesch
13 Dicembre	Aspetti biomolecolari delle neoplasie polmonari e implicazioni cliniche	Milo Frattini

Anno 2012

DATA	TITOLO	RELATORE
31 gennaio	Neoplasie della pelle: macroscopia e classificazione	Sandra Leoni-Parvex
13 marzo	Nevi Spitz: un aiuto dalla citogenetica molecolare?	Vittoria Martin
3 aprile	Vantaggi e svantaggi dei Tumor Board	Luca Mazzucchelli
24 aprile	Neoplasie linfoproliferative: classificazione, macroscopia e immunoistochimica	Elisabetta Merlo
15 maggio	Utilità delle analisi molecolari nelle neoplasie linfoproliferative	Milo Frattini
5 giugno	Cause degli errori in patologia	Lara Lunghi
26 giugno	Artefatti pericolosi	Luca Mazzucchelli
4 settembre	Neoplasie ginecologiche: macroscopia e classificazione	Tiziana Rusca
25 settembre	Epidemiologia delle neoplasie ginecologiche	Stefano Crippa

Partecipazioni a congressi e seminari

Congresso: 22nd IUBMB, 37th FEBS, from Single Molecules to System Biology, Siviglia, Settembre 4-9, 2012. "Evidence of human sialidase NEU3 involvement in colorectal carcinogenesis" (Mozzi A, Forcella M, **Riva A,** Molinari F, Frattini M, Fusi P.). **Poster.**

Congresso: 77 th Annual Meeting of the Suisse Society of Pathology, Lucerna, Novembre 10-12, 2011. "Investigation of the role of NEU3 in colorectal carcinogenesis and in the prediction of efficacy of EGFR targeted therapies" (**Riva A,** Lampis A, Molinari F, Perrone F, Romanelli D, Forcella M, Mozzi A, Pilotti S, Monti E, Fusi P, Mazzucchelli L, Frattini M). **Presentazione orale.**

Simposio: "Melanoma: finalmente buone nuove". Lugano, Svizzera, 26.04.2012.

- "Epidemiologia del melanoma in Ticino". Dr. Andrea Bordoni, ICP, Locarno (Svizzera).
- "Dermatoscopia e istologia: due tecniche complementari". Dr. Claudio Clemente- Dr. Agostino Crupi, Casa di Cura S. Pio X, Milano (Italia).
- Citogenetica interfasica (FISH) nella diagnostica del melanoma". Dr.ssa Vittoria Martin, ICP, Locarno (Svizzera).
- "Linfonodo sentinella: update". Dr.ssa Sandra Leoni-Parvex, ICP, Locarno (Svizzera).
- "Analisi di patologia molecolare: BRAF e oltre". Dr. Milo Frattini, ICP, Locarno (Svizzera).
- "Predisposizione genetica al melanoma maligno nella Svizzera italiana". Dr.ssa Cristina Mangas, Servizio Dermatologia EOC, Bellinzona (Svizzera).

Simposio: "Carcinomi polmonari: ricerca di algoritmi per un miglior trattamento del paziente". Lugano, Svizzera, 01.12.2011.

- "Verso lo screening per il carcinoma del polmone?". Dr. Francesco Zappa. Clinica Luganese SA, Lugano (Svizzera).
- "La diagnosi patologica nei carcinomi polmonari: passato, presente e futuro". Dr.ssa Elena Passega-Sidler, ICP, Locarno (Svizzera).
- "Test molecolari: quali effettivamente utili?". Dr.ssa Francesca Molinari, ICP, Locarno (Svizzera).
- "Trattamenti chemioterapici standard: update". Dr. Luciano Wannesson, IOSI, Bellinzona (Svizzera).
- "Targeted terapie: EGFR e oltre". Dr. Federico Cappuzzo, Istituto Toscano Tumori, Livorno (Italia).

Seminario: "Le interazioni farmacologiche in oncologia". *Dr. Med. Dario Caronzolo, Dr. Med.Marco Bissig*. IOSI, Bellinzona (Svizzera) 03.10.2012.

Seminario: "Screening programs for early detection of colorectal cancer". *Dr. Carlo Senore, Ospedale S. Giovanni Battista, Torino*. IOSI, Bellinzona (Svizzera) 19.09.2012.

Seminario: "The introduction of biomarkers in early clinical drug development in oncology". *Dr. Josep Tabernero, Head Dept. Medical Oncology, Vall d'Hebronn University Hospital, Barcellona.* IOSI, Bellinzona (Svizzera) 13.06.2012.

Seminario: "Selection and adaptation during systemic cancer progression". *Prof. Christian Klein, University of Regensburg.* IOSI, Bellinzona (Svizzera) 16.05.2012.

Seminario: "Radiochemotherapy in lung cancer". *Prof. Branislav Jeremic, Applied radiation Biology and Radiotherapy, IAEA, Vienna*. IOSI, Bellinzona (Svizzera) 18.04.2012.

Seminario: "Rational design of new therapeutic agents for melanoma treatments". *Prof. Olivier Michielin, CePO-CHUV, Ludwig Institute for Cancer Research, Losanna*. IOSI, Bellinzona (Svizzera) 15.02.2012.

Seminario: "New biologic and therapeutic aspects in malignant melanoma". *Prof. Reinhard Dummer, Department of Dermatology, University Hospital, Zurigo*. IOSI, Bellinzona (Svizzera) 18.01.2012.

Pubblicazioni

"The clinical implications of HER2 gene copy number in metastatic colorectal cancer patients treated with anti-EGFR monoclonal antibodies." Vittoria Martin[1], Lorenza Landi[2], Francesca Molinari[1], George Fountzilas[3], Ravit Geva[4,5], **Alice Riva[1]**, Piercarlo Saletti[6], Sara De Dosso[6], Alessandra Spitale[7], Sabine Tejpar[4], Konstantinos T. Kalogeras[3], Luca Mazzucchelli[1], Milo Frattini*[1], Federico Cappuzzo*[2].
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