Review Article

Copy number gains of FGFR1 and 3q chromosome in squamous cell carcinoma of the lung

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Abstract: Squamous cell carcinoma of the lung (SQCCL) remains a leading cause of cancer-related death. Unlike non-smoker adenocarcinoma of the lung, where highly efficient tyrosine kinase inhibitors are available for treating mutant EGFR or ALK-rearranged, no targetable biomarkers are available for SQCCL. The frequent and focal amplification of FGFR1 has generated great expectations in offering new therapeutical options in case of 16-22% of SQCCL patients.

Broad 3q chromosome amplification is widely recognized as the most common chromosomal aberration found in SQCCL, where PIK3CA, SOX2, ACK1, PRKCI, TP63, PLD1, ECT2, and others genes are located. Although SOX2 has been postulated as a key regulator of basal stem cells transformation and tumor progression, it seems to confer a good prognosis in SQCCL. It is known that each patient might carry a different length of 3q chromosome amplicon. Thus, we suggest that the number and the biological importance of the genes spanned along each patient's 3q amplicon might help to explain inter-individual outcome variations of the disease and its potential predictive value, especially when relevant oncogenes such as those mentioned above are implicated.

Currently, there is no clinical predictive data available from clinical trials. In this review, we have focused on the potential role of FGFR1 in SQCCL prognosis. Additionally, we have explored recently available public data on the comprehensive genomic characterization of SQCCL, in relation to the protein-coding genes that have a strong gene copy number - mRNA correlation in 3q chromosome, that were previously described as potential driver oncogenes or its modifiers in SQCCL.

Key Words: FGFR1; Squamous cell carcinoma; 3q chromosome; amplification; lung cancer

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Despite the decrease in the incidence of squamous cell carcinoma of the lung (SQCCL) in the last decades, it still represents 20-30% of non-small cell lung cancer (NSCLC) (1). Unlike non-smoker lung adenocarcinoma, where strong biomarkers of response to specific tyrosine kinase inhibitors (TKI) (such as activating mutations of EGFR or ALK rearrangements) are available, in SQCCL actionable alterations have only been partially characterized in recent years, without any breakthrough in treating such tumor entities (2).

Gene copy number (GCN), like other genetic structural variations, represents an event of strong evolutionary pressure within both normal cells and particularly in cancer cells, where genomic instability it is a hallmark. GCN gains, such as gene duplication or amplification, can cause an increase in protein levels. Nowadays, there are three

molecular mechanisms that can potentially produce a gene amplification, including the double-stranded DNA repair pathways: non-homologous end-joining (NHEJ), non-allelic homologous recombination (NAHR) (3,4), and DNA re-replication. In DNA re-replication, license control of replication is lost and a single DNA molecule is replicated more than once, triggering GCN gains, amplification, genomic instability and tumorigenesis (5).

Fibroblast growth factor receptor 1 (FGFR1) is one of the four family members of the FGFR of transmembrane tyrosine kinase receptors (TKR) involved in regulation of embryonic development, differentiation and cell proliferation (6-8). The functional validation of FGFR1 gene amplification in SQCCL was initially described by Weiss *et al.* Their work placed this histological-tumor subtype on the edge of the wave, identifying new therapeutic options that could change the management of SQCCL patients (9).

Broad amplification at 3q chromosome is the most frequent chromosomal alteration in SQCCL tumors. It was initially reported using fluorescent in situ hybridization (FISH) (10). It is known that increasing frequency of 3q amplification can be found from dysplasia to metastatic squamous lesions (11). Moreover, the potential epidemiological relationship of 3q amplification and tobacco consumption has been suggested (2). A recent comprehensive genomic characterization of SQCCL reported that 3q amplicon covers 3q13 to 3q29 (12). They also showed a correlation of GCN and mRNA levels at single-gene resolution.

This review highlights the recent findings on the prognostic and/or predictive value of FGFR1, as well as other important genes targeted by the 3q chromosome amplification in SQCCL.

FGFR1 amplification

FGFR is a family of receptors tyrosine kinases (RTK) consisting of four family members (FGFR1-4). FGFR1, like other RTKs, has an extracellular domain, a transmembrane domain and an intracellular domain, where the catalytic tyrosine kinase domain is located. The FGFR1 gene resides at 8p12 cytoband and spans a genomic DNA fragment of 57.7 Kb in length. Upon receptor activation, it promotes cell proliferation, angiogenesis, survival and apoptotic resistance through the PLCγ/PKC, RAS/MAPK and PI3K-AKT pathways (13). The oncogenic potential of activated FGFR1 represents an attractive therapeutic target that is currently being clinically tested.

The seminal work of Weiss *et al.* (9), demonstrated a growth dependency of a subset of SQCCL based on FGFR1 amplification that was abrogated both in lung cancer cell lines and in NCI-H1581 mice xenografts by PD173074, a specific TKI. No activating mutations were found. Twenty-two percent of squamous lung cancer tumors were carriers of FGFR1 focal amplification, as detected by FISH. Further studies confirmed that the percentage of amplification ranges from 16-22% (14-16) and independent *in vitro* studies confirmed that FGFR1-amplified cells are vulnerable when treated with a specific TKI (17). FGFR1 has also been reported to be amplified in other cancers, including 17.4% oral squamous cell carcinoma (18), 6% of esophageal squamous cell carcinoma (19), 10-17% of breast (20,21), 7.8% of ovarian (22,23), 3.4% of bladder (24) and 9% of prostate cancer (25).

Heist *et al.*, in a retrospective cohort of 226 SQCLC, where almost 70% of the patients were staged as IA-IIB, detected 16% of FGFR1 amplification. They measured gene copy number by FISH , using for the threshold of gene amplification a FISH ratio equal to 2.2 or higher (14). In this study, amplification of FGFR1 was not correlated with age, sex, stage or smoking history. They found no correlation with overall survival. On the other hand, Weiss *et al.* reported a trend towards inferior survival among patients amplified for FGFR1 (9). In a recent work carried out by Kim *et al.* reported that patients, carriers of FGFR1 amplification, had significantly shorter disease-free survival and overall survival than diploid patients (wild type), regardless of sex, smoking status, adjuvant therapy and pathologic stage. These findings are in contradiction to those previously published by Heist and Weiss, and suggest FGFR1 amplification is an independent prognostic marker in this cohort of patients. Furthermore, in the same study, a positive association of FGFR1 amplification and smoking habit, in a dose-dependent manner, was reported. An interesting observation is that none of the 37 patients classified as never-smokers were carriers of amplified FGFR1 (26). Recently, a 100% concordance of FGFR1 amplification between primary SQCCL tumors and their lymph node metastatic tissue was described, suggesting an important role for FGFR1 in tumor prognosis and progression. So further studies are needed to validate whether the prognostic impact of FGFR1 amplification is a population-based phenomena or not (16).

Due to the important biological impact of FGFR

Table 1 Selected FGFR inhibitors currently used in clinical development and/or evaluation

DRUG	Company	TARGETS	Clinical development stage
Small-Molecule TKIs			
Vargalef (BIBF1120)	Boehringer Ingelheim, Novartis	FGFRs, VEGFR and PDGFR	III
Ponatinib (AP24534)	Ariad	FGFR, VEGFR and IGF-1R	I
Dovotinib (TKI258)	Novartis	FGFRs, VEGFRs, KIT, FLT3, CSFR and PDGFRs	III
Brivanib (BMS582664)	Bristol Myers Squib	VEGFRs and FGFRs	II
AZD4547	Astra Zeneca	FGFRs	I/II
Cediranib (AZ2171)	Astra Zeneca	VEGFRs, FGFRs and KIT	III
TSU68 (SU668)	Taiho Pahrmace	FGFRs, VEGFR and PDGFR	II
E7080	Eisai	FGFRs, VEGFR and PDGFR	II
E3810	Ethical Oncology Science	FGFRs, VEGFR	I
BGJ398	Novartis	FGFRs	I
RG1507	Roche, Genmab	FGFRs, VEGFR and PDGFR	II
LY2874455	Lilly	FGFRs	n/a
FGFR antibodies			
Figitumumab	Pfizer	FGFR, VEGFR and IGF-1R	III
Cixutumumab	ImClone Systems	FGFR, VEGFR and IGF-1R	II
AMG479	Amgen	FGFR, VEGFR and IGF-1R	II/III
BIIB022	Biogen Idec	FGFR, VEGFR and IGF-1R	I/II
FP1039 (Fusion protein)	Five Prime	FGFR1	I/II
R3Mab	Genectech	FGFR3	n/a

Abbreviations: FGFRs, fibroblast growth factor receptors; VEGFRs, vascular endothelial growth factor receptor; PDFRs, platelet derived growth factor receptor; IGF-1R, Insulin Growth factor-1 receptor; KIT, mast/stem cell growth factor receptor; FLT3, fms-like tyrosine Kinase receptor 3; CSFR, colony stimulating factor receptor; n/a, not applicable

activation in tumor cell growth, survival, tumor angiogenesis, progression and metastasis, the development and clinical testing of anti-FGFR compounds are currently major areas of research. There has been a great expectation as some reports have suggested FGFR1 amplification as a predictive biomarker of specific TKIs. There are two different types of FGFR inhibitors under development: small TKI molecules and ligand-competitor antibodies (see *Table 1*). Most of the small molecules exert their biological activity by binding into the ATP-binding pocket. This prevents either auto-phosphorylation of the receptor or proliferative signal transduction through transphosphorylation of receptor-dimers and their downstream adaptor proteins such as FSR2 (17,27). A clinical trial with BIBF1120, which inhibits FGFR1, will be developed in the Netherlands and in Spain in the second line treatment of SQCCL patients with FGFR1 amplification. Double methodological validation of FGFR1-amplified tumors will be carried out by FISH and multiplex ligation-dependent probe amplification (MLPA).

Taking advantage of what we have learned from gastrointestinal stromal tumors treated with imatinib/sunitinib (28,29), as well as from the history of erlotinib/gefitinib or crizotinib in lung cancers carriers of mutant EGFR (30,31) or ALK-rearrangements (32) respectively, we will need to identify the mechanisms of intrinsic, adaptive and acquired resistance to TKI treatment, as quickly as possible, and how to revert them clinically. The priority should be to analyze the presence of gain-of-functional mutations, amplification or overexpression of RTKs that activates redundant pro-survival pathways which bypass the drugged one (33,34). In addition, alterations in apoptotic pathways have also been demonstrated a key role in TKI resistance, and thus need to be analyzed (35-38).

Currently, fluorescent in situ hybridization (FISH) is the standard method available for identification of gene amplification among cancer patients. The previous

experience from ERBB2 in breast cancer has shown that a key point was the inter-laboratories standardization of FISH criteria (39,40). Recently it has been reported in a cohort of 307 squamous lung carcinomas a reference guide to classify the tumor entities with respect to their FGFR1 gene status by FISH (41). The authors defined low-level amplification by ≥5 FGFR1 signals in ≥50% of tumor cells, whereas high-level amplification is defined by an FGFR1/centromere 8 (CEN8) ratio ≥2.0, or by an average number of FGFR1 signals per tumor cell nucleus ≥6, or by the percentage of tumor cells containing ≥15 FGFR1 signals or large clusters ≥10%.

In order to establish an appropriate GCN threshold correlation between FGFR1 gene dosage and drug response in SQCCL patients, we propose to measure FGFR1 gene status by FISH along with, a secondary independent quantification of FGFR1 gene copy number by MLPA. In addition to clarify how FGFR1 amplification is translated at active-protein levels, we recommend measuring phospho FGFR1 and phospho FSR2 as indicators of FGFR1 signal transduction activity (17,27).

3q amplification

Over the recent decades, due to the great technical advancement in the field of molecular biology, there has been vast improvement towards the genetic characterization of tumors, in an effort to understand how their biology can be targeted to improve cancer patient care. One of the most frequent chromosomal aberrations found in NSCLC is the amplification at 3q chromosome, which can be present in up to 43% of cases. It can be found in squamous dysplasia, established carcinoma and also in metastatic tissue (42) and is suggested that 3q amplification frequency increases as disease progresses (43). It is known that each patient carries a different length of 3q chromosome amplicon (see *Figure 1*). We hypothesized that the number and the biological importance of the trapped genes in each patient's 3q amplicon might be helpful to explain the inter-individual differences in disease outcome or its response towards specific targeted therapy.

Only a few genes that are targeted by the 3q chromosome amplicon have been functionally validated as prognosis modifiers of cancer disease, and even fewer as biomarkers of cancer therapy. Among these genes are phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) (44-46), SRY-related HMG-box (SOX2) (47-50), tumor protein 63 (TP63) (42), atypical Protein kinase C iota (aPKCι) (51,52), eukaryotic translation initiation factor 4 gamma 1 (EIF4G1) (53,54), member of RAS oncogene family RAP2B, and others.

PIK3CA encodes for the p110α catalytic subunit of phosphatidylinositol (PI) 3-kinase. A broad range of cancer-related functions have been associated with its activation, such as cell proliferation, survival, oncogenic RAS signaling and transformation, making this an attractive target for therapeutic intervention. PIK3CA was found to be amplified in up to 45% of SQCCL cancer patients (55-59) and, due the strong correlation between PIK3CA amplification and its increased activity through its downstream effectors such as AKT and mTOR, this gene also appeared as an oncogene candidate (44). Abnormalities including mutations and amplification of PIK3CA/AKT/mTOR/PTEN are more common in SQCCL than in adenocarcinoma of the lung (60-62). Similar results have been showed by Spoerke *et al.* in their study where they have evaluated the candidate predictive biomarkers of sensitivity to select PI3K/mTOR pathway inhibitors in lung cancer patients. They suggests that different predictive biomarker strategies might be needed for both squamous and non-squamous patient populations, due to their alteration patterns and frequency (46).

The transcription factor TP63 (TP73L) is a homologue of p53 that functions by transactivating p53-targeted genes. The TP63 gene is expressed as multiple isoforms with different functions, including a full length (TAp63) and a truncated amino-deleted isoform ΔNp63, also called p40 (63). TAp63 can induce cell cycle arrest and apoptosis in response to DNA damage (64), whereas ΔNp63 has opposite functions because of its competition with p53, with respect to cell cycle arrest, mobility, invasion (epithelial–mesenchymal transition) and senescence. The ratio of TAp63 and ΔNp63 regulates chemosensitivity. ΔNp63a is the most commonly expressed TP63 isoform in squamous cell carcinoma together with TP63 amplification (65). Massion *et al.* reported that 88% of SQCCLs have TP63 amplification by FISH (42). As an interesting finding, they observed that TP63 amplification was an early event in the development of squamous carcinoma along with overexpression by IHC which results in better survival. ΔNp63 has been demonstrated as a more specific maker of squamous cell carcinoma than full length TP63, in the differential diagnosis in comparison with other lung histologies (66,67).

The SOX2 gene is a key transcription factor that coordinates embryonic development, differentiation and self-renewal of normal non-alveolar epithelium of the

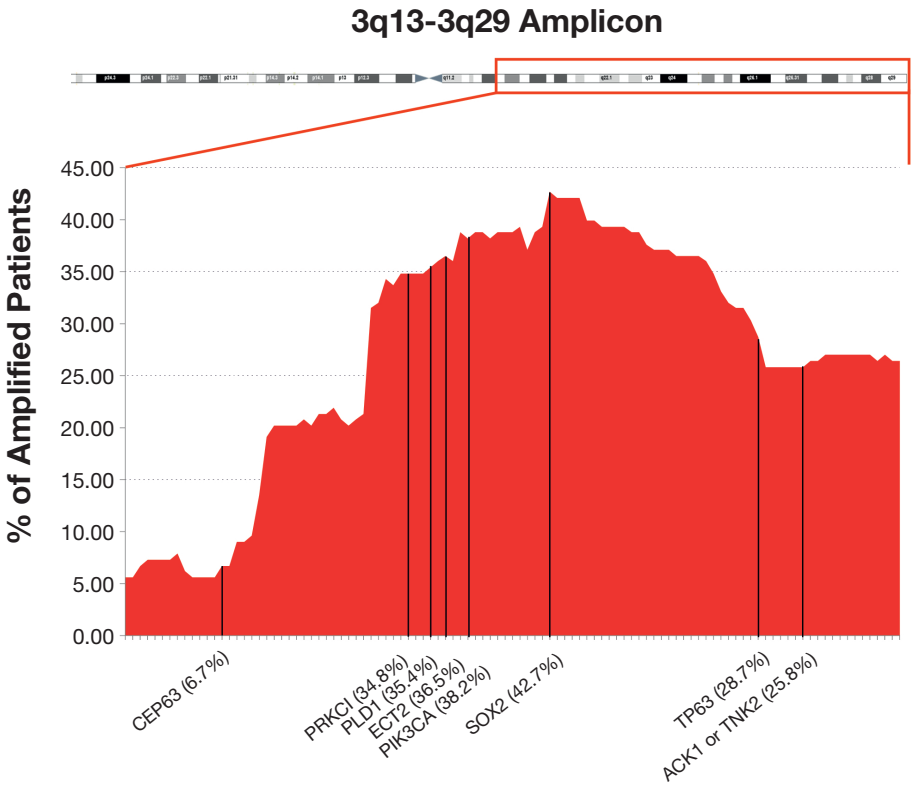

Figure 1 Percentage of patients, carriers of 3q chromosome amplification for each gene. A representative list of 3q chromosome region was sorted by chromosome position from 3q13 to 3q29

airway. SOX2 amplification has been reported in 43-60% (11,48,50,68) of SQCCL and in 27% of SCLC (69). The biological and clinical impact of SOX2 in lung cancer is reviewed by Karachaliou *et al.* (doi: 10.3978/j.issn.2218-6751.2013.01.01).

CEP63 (centrosomal protein 63 kDa) plays a role in DNA damage response. Following DNA double strand breaks (DSBs) formation, it is delocalized from centrosomes and recruits CDK1, a regulator mitotic entry of the cell (70,71).

We took advantage of a recent report where authors performed a high resolution genomic characterization of SQCCL by RNA-seq, gene copy number and mRNA expression analysis (12) that is publicly available at the cBio cancer genomics portal (72). In this section, we will summarize the recent evidence of selected 3q-resident genes, where gene amplification might explain its contribution to malignant transformation, tumor progression or its role as a biomarker for targeted therapies. From protein-coding genes located at 3q, we only selected those were having strong correlation of GCN and mRNA. We defined strong GCN-mRNA correlation for a given gene, when at least 50% of the amplified tumors expressed higher levels of mRNA than diploid tumors (see *Figure 2*).

Atypical protein kinase C iota (aPKCι)

aPKCι belongs to the atypical subgroup within the protein kinase C family of structurally related serine/threonine kinases. Different PKC isoenzymes are involved in different functions, such as: cellular differentiation, proliferation, polarity and apoptosis. Atypical PKCs, unlike most of the members of the family, can be activated independently of Ca^{2+}, diacyglycerol or phosphatidylserine (73). High aPKCι expression has been found in several human tumors, including squamous carcinomas of head and neck (64), esophageal (74,75) and lung (52), but also in lung adenocarcinoma (76). Recent data suggests that aPCKι activity is required by the oncogenic RasG12D mice model to progress from bronchial hyperplasia to lung tumor (77). In the same study, bronchoalveolar stem cells that lacked Prkci, the mice gene that encodes for aPKCι, were unable to transform neither *in vitro* nor *in vivo*.

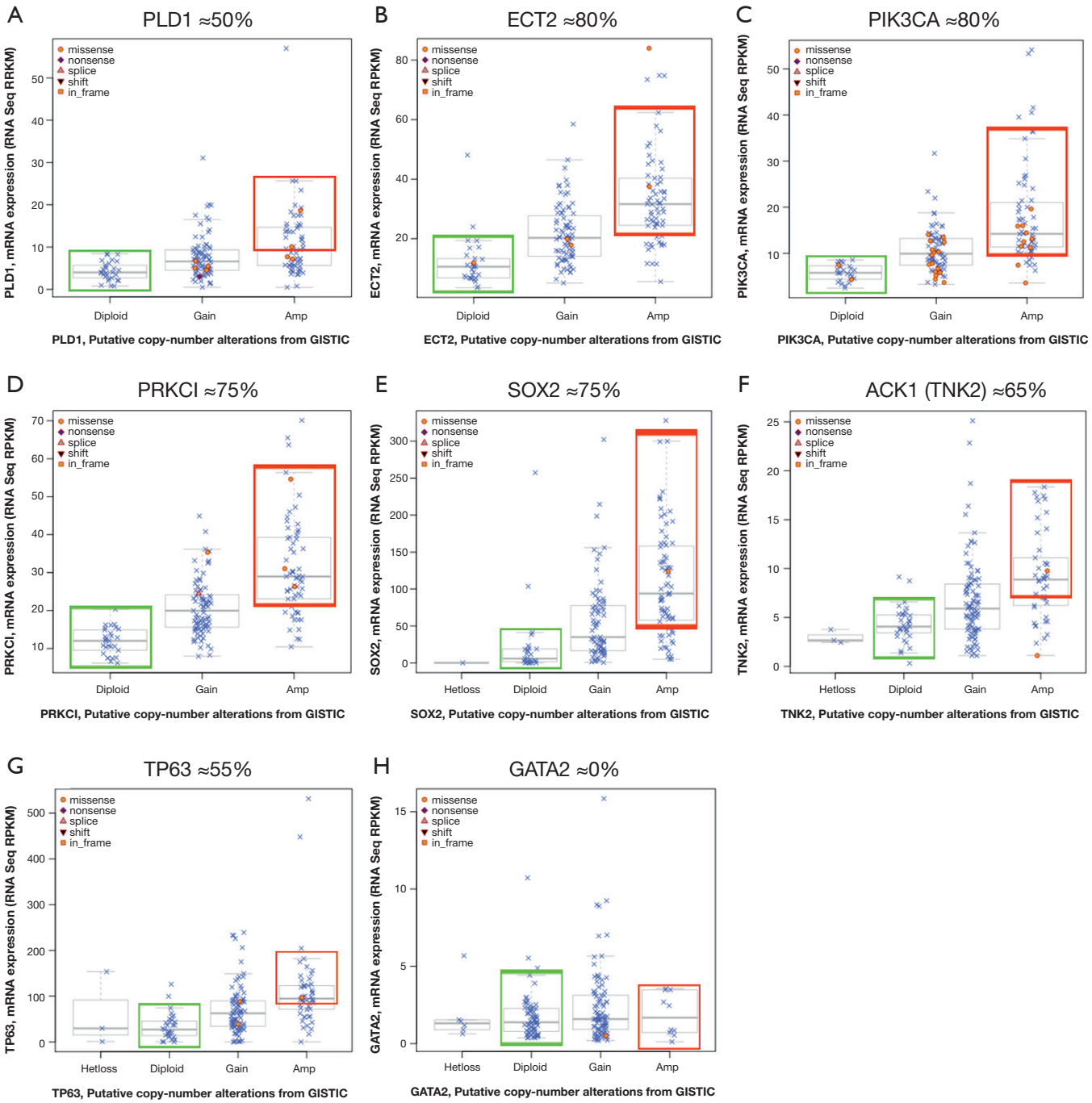

Figure 2 Boxplots of mRNA expression vs. gene copy number in SQCCL selected oncogenes. Only protein-coding genes were considered for screening as candidate genes. Gene data was retrieved from the cBio portal (http://www.cbioportal.org/public-portal/) based on their GCN- mRNA correlation and previous available bibliography. Those genes in which ≥50% of the amplified tumors expressed higher mRNA levels than diploid were selected for further bibliographic review. A-G, selected genes showing an strong correlation of GCN and mRNA expression levels. H, an example of a discarded gene due to its low correlation value

aPKCι interacts with PAR6α, forming a complex that triggers the activation of RAC1-PAK-MEK-ERK pro-survival pathway. Interestingly in NSCLC, the ECT2 oncogene, which also localizes at 3q amplicon, is mislocalized in the cytoplasm, where it is a target of phosphorylation at Thr-328 by aPKCι (78) for a proper oncogenic signaling through the RAC1 small GTPase pathway (79).

Taking into consideration, the importance of aPKCι in KRAS-mediated lung tumors, the prognostic and/or predictive role of PRKCI amplification and aPKCι overexpression needs to be evaluated in oncogene "addicted" lung tumors, such as lung adenocarcinoma induced by EGFR-activating mutations or oncogenic rearrangements of ALK, where targeted therapies have a strong impact on patient survival and quality of care. Of course, it might be also interested to address the same question in SQCCL carriers of FGFR1 amplification treated with FGFRs inhibitors.

Activated CDC42 kinase 1 (ACK1)

ACK1, also known as TNK2, is a non-receptor tyrosine and serine/threonine protein kinase which functions as transducer of multiple ligand-activated RTKs including EGFR (80,81), AXL (82), MERTK (53), HER2 (83) and PDGFR (84) by activating cytosolic or nuclear effectors such as AKT and AR respectively to promote cell growth and survival (85,86). EGF ligand stimulation activates the ACK1 activity, which at the same time prevents EGFR from ubiquitination (87). AKT activation by ACK1 happens in a PI3K-independent manner. When phosphorylated by ACK1 at Tyr-176, unlike the PI3K-activated AKT, it is confined to the membrane phosphatidic acid phospholipid. Once the phospho-activated AKT/ACK1 complex is located at the plasma membrane, it then translocates into the nucleus where it phosphorylates FoxO3a, preventing the expression of the BIM-1 pro-apoptotic gene, the GADD45 DNA repair gene and p21 and p27 inducers of cell cycle arrest. Moreover it can also activate the mitotic progression (88). In addition, the E3 ubiquitin ligase Nedd4-2 is a negative regulator of ACK1 when co-expressed (87,89), and can be rescued by treatment with MG132, a proteasomal inhibitor. Xenografts of prostate LNCaP cells are usually poorly tumorigenic in nude mice. But when LNCaP cells expressing a constitutively activated ACK1 were engrafted into nude mice, they rendered very large tumors within the first 24 days after injection. In prostate cancer, activated ACK1, phosphorylates androgen receptor (AR) either at Tyr-267 or Tyr-363 led to the nuclear translocation of AR/ACK1 complex, thus activating the transcription of AR target genes such as prostate-cancer proteins: prostate-specific antigen (PSA) and HK2, independently of androgen or testosterone, the Androgen receptor ligands (83). Interestingly, a hallmark of prostate cancer progression implies the acquisition of an androgen-resistant phenotype, which might be explained in some cases by the AR estrogen-independent activation by ACK1.

Conclusions

Taking into consideration, the potential biological and medical impact of FGFR1, its activation turned to be a major area of research interest. Although prognostic data on FGFR1 has only recently been reported, the results are contradictory. Larger studies are needed to clarify its prognostic role. Furthermore, FGFR1 inhibitors have entered clinical trials, and over the next few years its predictive role with targeted TKIs will be definitely clarified.

On the other hand, finding new predictive biomarkers in highly genetic heterogeneous tumors such as SQCCL might be challenging because of the coexistence of multiple driver oncogenes, both in the same cellular clone or in different ones. An example might be the 3q chromosome amplification in SQCCL.

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