Analysis of the intra-tumor heterogeneity and consistency between *FGFR1* gene amplification and protein expression in squamous cell lung cancer

Monika Skupinska[1]*, Paulina Jagus[2]*, Paulina Grygielewicz[1,3], Agata Mikolajczyk[1], Ewa Szczepulska[4], Adriana Rozy[2], Renata Langfort[4], Joanna Chorostowska-Wynimko[2], Maciej Wieczorek[1], Aleksandra Stanczak[1+]

* both authors contributed equally, +corresponding author: aleksandra.stanczak@celonpharma.com

[1]Innovative Drugs R&D Department, Celon Pharma S.A., Lomianki/Poland, [2]Department of Genetics and Clinical Immunology, National Institute of Tuberculosis and Lung Diseases, Warsaw/Poland, [3]Postgraduate School of Molecular Medicine, Zwirki i Wigury 61, 02-091 Warsaw/Poland [4]Department of Pathomorphology, National Institute of Tuberculosis and Lung Diseases, Warsaw/Poland

Introduction

Fibroblast growth factor receptors (FGFRs) induce intracellular signaling networks that tightly regulate key biological processes, such as cell proliferation, survival, migration, and differentiation. Preclinical data have shown that inhibition of fibroblast growth factor receptor 1 (FGFR1) could be a promising therapy for lung tumors with *FGFR1* amplification or expression [1]. The candidate predictive biomarkers *FGFR1* amplification and protein overexpression has been reported respectively in 10-20% or 10-41% patients (pts) with squamous cell lung carcinoma (SqCLC) [2]. Intra-tumoral molecular heterogeneity is well known phenomenon in lung cancer, nevertheless its prevalence has not been analyzed for *FGFR1* amplification nor expression yet. Therefore, there is an urgent need to assess relationship between *FGFR1* amplification and expression, as well as the heterogeneity of their intratumoral distribution as the potential confounding factors for testing reliability.

Materials and Methods

Patients' characterictics

Table 1. Clinicopathological characterictics of patients

Number of cases		20
Age in years (median, range)		66; 54-82
Gender	female	5
	male	15
Smoking history	smoker	9
	former smoker	5
	no information	6
Stage	I	8
	II	7
	III	5
Differentiation	G1	1
	G2	17
	G3	2

The study was approved by local ethical committee.

Materials

3 to 5 Formalin-Fixed, Paraffin-Embedded (FFPE) sections from different regions of each of 20 SqCLC tumors were analyzed.

Fluorescence *in situ* hybridization (FISH)

FGFR1 gene copy number was assessed by FISH method using probes specific for the 8p12 locus and the chromosome 8 centromere (CEN8) ZytoLight SPEC *FGFR1*/CEN 8 Dual Color Probe (ZytoVision). Amplification was defined as previously described by Schultheis *et al.* [3]. Criteria of *FGFR1* amplification were as follows: *FGFR1*/CEN8 ≥2.0 or the average number of *FGFR1* signals per cell ≥6 or >10% of tumor cells containing ≥15 *FGFR1* signals.

Immunohistochemistry (IHC)

FGFR1 protein expression was determined by immunohistochemistry. Tissue slides were subjected to antigen retrieval in Target Retrieval Solution, pH 9 (DAKO) with PT Link (DAKO). Tissues were incubated with anti-FGFR1 rabbit monoclonal antibody (Cell Signaling Technology, clone D8E4). Detection was done with EnVision TM+ system (DAKO). Expression was defined as staining intensity 2+ or 3+ (graded from 0 to 3+) in >1% of the cancer cells.

Heterogeneity

For the heterogeneity analysis only patients with >4 slides per tumor were taken into account (15/20 pts). Different definition of heterogeneity was considered:

- definition I - tumor was classified as heterogeneous for FISH or IHC when >25% of slides showed different results;
- definition II - tumor was classified as heterogeneous for FISH or IHC when result from at least one of slide was different than others.

Statistical analysis

Statistical calculation of correlation between FISH and IHC results (19/20 pts) was performed using the GraphPad Prism software version 5.03. using Spearman test.

Results

FGFR1 Amplification and Expression Patterns

FGFR1 amplification was observed in 6/20 (30%) SqCLC tumors. The average *FGFR1* gene copy number per cell ranged from 1.9 to 10.9 (mean: 4.6) and the mean *FGFR1/CEN8* ratio was 2.3 (range: 0.7–3.7). The mean content of tumor cells with ≥15 *FGFR1* copies was 2.2%. In IHC(+) tumors (5/20, 25%) the percentage of stained cancer cells with intensity ≥2 was low. Moreover, only 10/78 slides contained more than 10% of stained cancer cells.

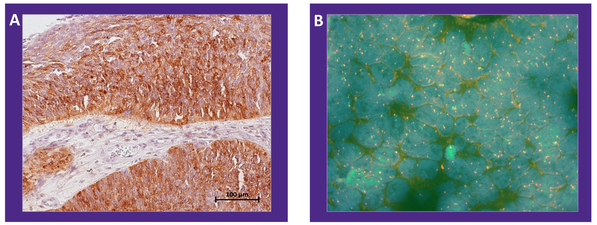

Figure 1. Representative examples of FGFR1 expression and amplification in SqCLC patients. (**A**) Strong cytoplasmic and membrane staining (IHC 3+; pt no. 20); (**B**) *FGFR1* amplification (pt no. 8; green signal *FGFR1*, orange signal *CEN 8*)

Table 2. Representative examples of FISH and IHC results. The parameters fulfilling the criteria for *FGFR1* amplification are shown in violet, for FGFR1 expression in orange.

tumor no.	slide no.	average FGFR1/CEN8 ratio	average number of FGFR1 signals per nucleus	percentage of tumor cells containing ≥15 FGFR1 signals	IHC score (0- 3+)
3	1	3.0	8.2	13.3	1+
	2	3.0	6.1	5.0	1+
	3	3.4	7.2	8.3	1+
	4	3.7	8.7	13.3	1+
5	1	0.9	2.4	0.0	0
	2	0.9	2.9	0.0	0
	3	0.9	2.6	0.0	0
	4	1.0	2.4	0.0	0
8	1	1.7	9.2	11.7	2+
	2	1.7	9.3	20.0	3+
	3	2.1	10.9	26.6	2+
	4	2.2	8.1	6.7	2+
	5	1.5	7.9	3.3	3+
15	1	0.9	2.8	0.0	2+
	2	0.9	2.6	0.0	3+
	3	0.9	2.4	0.0	0
	4	0.8	3.2	0.0	1+
20	1	1.8	3.6	1.7	3+
	2	1.3	3.8	0.0	3+
	3	1.8	3.7	0.0	3+
	4	1.5	3.5	0.0	3+

Correlation between amplification and expression of FGFR1

The FISH and IHC results were consistent in 52% SqCC pts (n=19), including 1/19 (5.2%) double-positive and 9/19 (47.3%) double-negative tumors. In 9/19 pts results were discordant: 5/19 (26.3%) IHC(-) FISH(+), while in 4 (21%) pts IHC(+) FISH(-). *FGFR1* amplification did not correlate with protein expression (P=0.543; r=-0.149) for 19 SqCLC tumors .

Results

Intra-tumoral Heterogeneity

62 FFPE samples from 15 SqCLC patients were analyzed for tumor heterogeneity.

Table 3. Summary of FISH and IHC assays' results for all samples analyzed towards heterogeneity.

tumor no.	IHC slide no. 1	2	3	4	5	IHC definition I: >25% of slides shows different results	IHC definition II: result from at least one of slide is different than others	FISH slide no. 1	2	3	4	5	FISH definition I: >25% of slides shows different results	FISH definition II: result from at least one of slide is different than others
1	(-)	(-)	(-)	(-)		IHC (-)	IHC (-)	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
3	(-)	(-)	(-)	(-)		IHC (-)	IHC (-)	(+)	(+)	(+)	(+)		FISH (+)	FISH (+)
5	(-)	(-)	(-)	(-)		IHC (-)	IHC (-)	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
6	(+)	(-)	(-)	(-)		IHC (-)	heterogeneous	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
8	(+)	(+)	(+)	(+)	(+)	IHC (+)	IHC (+)	(+)	(+)	(+)	(+)	(+)	FISH (+)	FISH (+)
9	(-)	(-)	(-)	(-)	(-)	IHC (-)	IHC (-)	(+)	(+)	(+)	(+)	(+)	FISH (+)	FISH (+)
12	(-)	(-)	(-)	(-)		IHC (-)	IHC (-)	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
13	(+)	(-)	(-)	(-)		IHC (-)	heterogeneous	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
14	(-)	(+)	(+)	(+)		IHC (+)	heterogeneous	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
15	(+)	(+)	(-)	(-)		heterogenous	heterogeneous	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
16	(-)	(-)	(-)	(-)		IHC (-)	IHC (-)	(-)	(+)	(-)	(-)		FISH (-)	heterogeneous
17	(-)	(-)	(+)	(-)		IHC (-)	heterogeneous	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)
18	(-)	(-)	(-)	(-)		IHC (-)	IHC (-)	(+)	(+)	(+)	(+)		FISH (+)	FISH (+)
19	(-)	(-)	(-)	(-)		IHC (-)	IHC (-)	(+)	(+)	(+)	(+)		FISH (+)	FISH (+)
20	(+)	(+)	(+)	(+)		IHC (+)	IHC (+)	(-)	(-)	(-)	(-)		FISH (-)	FISH (-)

(+) positive slides with FGFR1 expression or amplification; (-) negative slides without FGFR1 expression or amplification;
IHC (+) - FGFR1 expression of tumor (orange) ; FISH(+) - *FGFR1* amplification of tumor (violet)

According to definition I, *FGFR1* amplification was homogeneous in all pts (15/15), while heterogeneity of protein expression was confirmed in 1/15 tumors.
In contrast, according to definition II, heterogeneity was observed in tumor derived from 1 pt (1/15) with *FGFR1* amplification and in 5 pts with FGFR1 expression (5/15).

Conclusions

Our study demonstrated relative SqCLC tumor homogeneity in terms of *FGFR1* amplification and expression. However, tumor molecular heterogeneity strongly depended on the adopted definition type. *FGFR1* amplification did not correlate with protein expression. Therefore, more detailed evaluation of both biomarkers *FGFR1* amplification and protein expression regarding their predictive diagnostic value towards anti-FGFR therapy is needed.

References

[1] Tiseo M. *et al*. FGFR as potential target in the treatment of squamous non small cell lung cancer. *Cancer Treat Rev* (2015) 41, 527-539
[2] Touat M. *et al*. Targeting FGF signaling in Cancer. *Clin Cancer Res,* (2015) 21, 2684-2694
[3] Schultheis A.M. *et al.* Fibroblast growth factor receptor 1 (FGFR1) amplification is a potential therapeutic target in small-cell lung cancer. *Modern Pathology* (2014) 27, 214–221