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The frequency of EGFR gene mutations in a cohort of patients from Romania and their association with PD-L1 expression level and ALK rearrangements

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ABSTRACT

Background: The mortality rate linked to NSCLC cancer has notably decreased in recent years, primarily due to refined diagnostic techniques. This retrospective study aims to offer new insights into the frequency of EGFR gene mutations in Romanian NSCLC patients, examining potential associations or exclusions with ALK rearrangements and elevated PD-L1 expression level and seeks to contribute crucial insights into molecular marker alterations associated with NSCLC, advancing our understanding of targeted therapy prospects for oncology patients diagnosed with NSCLC in Romania.

Methods: DNA was extracted from the FFPET sections using the DNA Sample Preparation kit from Roche Diagnostics while the EGFR mutation detection test was performed using Real-Time PCR methods. PD-L1 expression levels and ALK rearrangements were immunohistochemically assessed.

Results: Among the 453 patients , 42 displayed EGFR gene mutations. The most prevalent mutation was Ex19Del, observed in 3.5% of cases, followed by the L858R substitution (2.9%). A noticeable elevation of PD-L1 expression level was observed on average when comparing patients EGFR Wild-Type with patients with EGFR gene mutations (40.37% versus 26.13%). The association of the L858R mutation and positive ALK was observed in one patient in our study cohort.

Conclusions: The study reveals a significantly higher prevalence of EGFR gene mutations among females and non-smokers. EGFR mutations were exclusively identified in patients with lung adenocarcinoma. This study data act as a catalyst for future investigations into resistance mechanisms to anti-EGFR TKIs in NSCLC patients in Romania and the prevalence of EGFR gene mutations associated with this phenomenon.

Keywords: ALK, EGFR gene, NSCLC, PD-L1, Real-Time PCR

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INTRODUCTION

Lung cancer persists as a prominent malignancy, even among non-smokers. Non-Small Cell Lung Cancer (NSCLC) stands out as the most commonly diagnosed histological subtype, demonstrating substantial morphological heterogeneity. The mortality rate linked to this cancer has notably decreased in recent years, primarily due to refined diagnostic techniques, notably the introduction of molecular tests with heightened accuracy and expedited execution.

Testing for activating mutations in the EGFR (Epidermal Growth Factor Receptor) gene, pivotal in signaling pathways governing cell growth, has paved the way for targeted lung cancer therapy. Predominantly affecting EGFR exons 18-21, encoding the tyrosine-kinase activating domain of the receptor, these mutations are the primary target of tyrosine-kinase inhibitors (TKIs) [1].

Another noteworthy molecular marker for targeted therapy (approved anti-ALK TKI) is the anaplastic lymphoma kinase (ALK). Fusion events involving ALK gene and EML4 or other partners result in the overexpression of the fusion protein. This overexpression initiates kinase domain multimerization, activating processes like cell proliferation, differentiation, and anti-apoptotic mechanisms [2].

In the realm of NSCLC treatment, immunotherapy is a valuable modality. While the human immune system recognizes and eliminates tumor cells, these cells can

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skillfully evade immune surveillance and action through immune checkpoints. A crucial target for this therapeutic approach is the PD-1 (Programmed death-1) receptor and its ligand, PD-L1 (Programmed death-ligand 1), potentially overexpressed, thwarting the activation of the anti-tumor immune response by inducing T cell apoptosis. Unlike EGFR gene mutations, PD-1/PD-L1 expression levels exhibit significant variability, influencing the likelihood of the response to immunotherapy [3].

Per the latest NCCN (National Comprehensive Cancer Network) guidelines for managing NSCLC patients, Category I assessment recommends testing for EGFR gene mutations, ALK rearrangements, and PD-L1 expression levels in advanced adenocarcinoma/NSCC-NOS (Non-Small Cell Carcinoma-Not Otherwise Specified)/large cell carcinoma cases. Conversely, squamous cell carcinoma patients undergo Category I assessment focusing on PD-L1 expression levels, with due consideration for EGFR gene mutation testing and ALK rearrangement presence [4].

In Romania, publicly available data for 2020 reveals 6567 new bronchopulmonary cancer cases, predominantly in males. Additionally, 18.9% of cancer-related deaths that year were attributed to lung cancer, with Romanian patient survival rates below the EU (European Union) average (source: Ministry of Health). A comprehensive national control program, addressing bronchopulmonary cancer management, including NSCLC, is underway at governmental level.

Presently, Romanian oncologists can adhere to ESMO (European Society for Medical Oncology) and NCCN guidelines. However, there is a lack of studies specific to the Romanian population, providing data on EGFR gene mutations, PD-L1 expression levels, or ALK gene rearrangements.

This retrospective study aims to provide novel insights of the frequency of EGFR gene mutations in Romanian NSCLC patients. It further investigates potential associations or exclusions with ALK rearrangements and elevated PD-L1 expression level. Consequently, the study endeavors to contribute pivotal insights into molecular marker alterations associated with NSCLC, thereby advancing our understanding of targeted therapy prospects for oncology patients diagnosed with NSCLC in Romania.

METHODS

Sample characteristics

The study examines FFPET (Formalin-fixed paraffin-embedded tissue) samples from 453 lung cancer patients at the GRAL Medical clinic, seeking consultation for EGFR gene mutations, ALK rearrangements, and PD-L1 expression levels between September 2019 and April 2023. The study cohort comprises exclusively Caucasian individuals, representing the Romanian population, with a nationwide geographic distribution. For all subjects, comprehensive clinical data, encompassing sex and age, were systematically collected. Additionally, when available, details regarding histopathological diagnosis, cancer staging, smoking status and initiated treatment (for EGFR-positive patients) were documented.

The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and received approval from the Ethics Committee of the GRAL Medical Clinic.

DNA isolation for EGFR gene mutation testing

DNA extraction was carried out using the DNA Sample Preparation kit (ROCHE Diagnostics), following the manufacturer's protocol. The assessment of DNA solution concentration and purity was performed using the Nanodrop spectrophotometer (ThermoFisher). Before initiating the DNA extraction process, the FFPET specimen from which the test will be performed undergoes evaluation by the pathologist. To be deemed eligible for testing, the sample must demonstrate a percentage equal to or greater than 10%. However, in specific cases, a percentage of 5-10% may be considered acceptable if the tumor cells are clustered, and macro-dissection of the FFPET specimen can be performed under histopathological control [5].

Real-Time PCR for EGFR testing

The identification of mutations within the EGFR gene was conducted utilizing the Real-Time PCR kit, cobas® EGFR Mutation Test v2 (ROCHE Diagnostics), in conjunction with the cobas z 480 analyzer (cobas 4800 application, PCR only). The kit utilizes specific primers, selectively amplifying predetermined segments within the EGFR gene (exons 18-21) rather than the entire gene. This targeted approach enables the detection of the following mutations:

- Exon 18: G719X (G719A, G719C, G719S);
- Exon 19: Deletions and complex mutations, defined as a combination of deletions and insertions (29 identifiable variants);
- Exon 20: S768I, T790M and insertions (5 identifiable variants);
- Exon 21: L858R, L861Q.

The manufacturer does not disclose the amplification protocol and stages, supplying amplification graphical displays solely for the positive and negative controls at the conclusion of the process [6].

PD-L1 immunohistochemistry testing

PD-L1 expression levels were immunohistochemically assessed using the SP263 antibody clone (Ventana) and the automated BenchMark ULTRA system (ROCHE Diagnostics), along with the Optiview DAB IHC Detection kit (ROCHE Diagnostics) for antibody detection. The SP263 antibody clone, vital for determining NSCLC patients eligibility for anti-PD-L1 immunotherapy, exhibits high affinity for tumor cells through a rabbit monoclonal antibody. A negative control slide stained with Negative Control Rabbit Monoclonal Ig was concurrently included. The Optiview DAB IHC Detection kit, utilizing an indirect system (non-biotin-based), employs visualization through a chromogen hydrogen peroxide substrate (DAB), resulting in an easily detectable brown precipitate under an optical microscope. Pathologist-led slide interpretation was conducted [7, 8].

ALK immunohistochemistry testing

The assessment of ALK rearrangements via immunohistochemistry involved the use of the D5F3 Rabbit Monoclonal Antibody clone (Ventana) and the automated BenchMark ULTRA system (ROCHE Diagnostics), along with the Optiview DAB Detection and Amplification kit (ROCHE Diagnostics) for antibody detection. The D5F3 antibody clone was applied to determine the eligibility of NSCLC patients for anti-ALK targeted therapy, demonstrating specificity and sensitivity comparable to the FISH technique. The Optiview DAB Detection and Amplification kit (ROCHE Diagnostics) intensifies the staining of rabbit antibodies through the incorporation of copper compounds and HRP peroxidase (Horseradish Peroxidase). Slide interpretation was conducted by the pathologist [9, 10].

Statistical analysis

For the statistical analysis of study data and the exploration of associations or exclusions among the investigated parameters, IBM SPSS Statistics software for Windows, Version 29.0, was employed (Armonk, NY: IBM Corp). Nominal data were presented as both absolute frequencies and percentages, while continuous variables were expressed in terms of mean and standard deviation. The assessment of associations among categorical variables involved cross-tabulation and the $\chi 2$ (chi-squared) test. To compare means based on dichotomous variables in the study, the independent samples t-test was applied. A significance level of $p < 0.05$ was deemed indicative of statistical significance.

RESULTS

Clinical and pathological characteristics of the patients of the study

This study comprehensively analyzes a cohort of 453 lung cancer patients, predominantly with NSCLC, necessitating assessment of EGFR gene mutations, PD-L1 expression levels, and ALK rearrangements. Adenocarcinoma prevails as the primary histopathological diagnosis (48.1% of the entire sample), followed by squamous cell carcinoma (Table 1). Among the 34 patients for whom we are aware of the presence of other types of cancer, 17 had a different lung cancer form than NSCLC, while 16 exhibited non-pulmonary origin metastases. In accordance with international recommendations, these patients would not qualify for EGFR gene mutation testing, all testing negative for EGFR gene mutations [4].

The study cohort encompasses both genders, with males comprising 66.0% and females 33.8%. The mean age is 65.23 years (youngest: 30 years, eldest: 89 years), showing an average deviation of 9.53 years above or below the mean. Regarding smoking status, data are available for 91 patients: 10 (2.2%) non-smokers, 55 (12.1%) smokers, and 26 (5.7%) former smokers. Of the 24 patients with information on occupational exposure to a toxic environment, 16 (3.5% of the total sample) have a history of working in such conditions. Among those with available information, the majority presented with cancer at stage IVB (14.1%), followed by stage IVA (8.2%) and stage IIIB (2.6%). The metastatic site is conclusively determined for 26 patients. Comprehensive clinical and pathological attributes of individuals in the study cohort are outlined in Table 2.

Table 1 . Histological subtypes observed in the patients of the study group

Histological subtype	Number of patients	Percentage from the entire sample (%)	Percentage from the valid data (%)
Adenocarcinoma	218	48.1	55.3
Adenosquamous carcinoma	12	2.6	3.0
Squamous cell carcinoma	101	22.3	25.6
Other NSCLC	29	6.4	7.4
Non-NSCLC	34	7.5	8.6
No available data	59	13.0	-
Total	453	100.0	-

Additional types of NSCLC included: non-small cell lung carcinoma with neuroendocrine differentiation, undifferentiated small cell carcinoma, large cell carcinoma, and non-small cell carcinoma favoring a pleomorphic subtype; all patients with these alternative NSCLC types tested negative for mutations in the EGFR gene.

Table 2. Clinical and pathological characteristics of the patients of the study

	Number of patients (Total=453)
Sex (male)	299 (66.0%)
Age (n=452)	65.2 (±9.5)
30-45	16
46-60	107
61-75	274
>75	55
Smoking status	
Never smoker	10 (2.2%)
Current smoker	55 (12.1%)
Ex-smoker	26 (5.7%)
Toxic work environment	
No	8 (1.8%)
Yes	16 (3.5%)
EGFR status	
Negative (Wild-Type)	391 (86.3%)
Positive	42 (9.3%)
EGFR mutation type	
DelEx19	16 (3.5%)
G719X	3 (0.7%)
insEx20	3 (0.7%)
L858R	13 (2.9%)
L861Q	4 (0.9%)
G719X+S7681	1 (0.2%)
L858R+insEx20	1 (0.2%)
L861Q+G719X	1 (0.2%)
PD-L status	
Negative	54 (11.9%)
Negative (+ immune cells)	183 (40.4%)
Positive	211 (46.6%)
PD-L1 expression level (n=183)	38.7 (±30.4)
ALK status	
Negative	409 (90.3%)
Positive	33 (7.3%)
ALK expression level (n=16)	51.0 (±41.6)
Clinical stage (NCCN)	
IA	5 (1.1%)
II	2 (0.4%)
IIB	3 (0.7%)
IIIA	6 (1.3%)
IIIB	12 (2.6%)
IIIC	9 (2.0%)
IVA	37 (8.2%)
IVB	64 (14.1%)
IVC	1 (0.2%)
Known metastatic site (n=26)	
Brain	7
Liver	5
Lymph node	4
Bone	5
Lung	5
Deceased (EGFR+ only)	
No	29 (6.4%)
Yes	12 (2.6%)

Age and the expression levels of ALK and PD-L1 are expressed as mean ± SD. Abbreviations: ALK- anaplastic lymphoma kinase, EGFR- epidermal growth factor receptor, NCCN- National Comprehensive Cancer Network

DISCUSSION

The distribution of mutations in the EGFR gene in the study cohort

According to 2021 statistics of the World Health Organization (WHO), lung cancer ranks as the second most frequently diagnosed malignancy in Romania, representing 12.3% of cases, following colorectal cancer at 13.1%. Particularly among male patients, lung cancer surpasses other diagnoses, accounting for 16.8% of cases. In both prevalence and mortality rate, lung cancer holds the foremost position in Romania [11]. Among the 453 study patients, 42 displayed EGFR gene mutations (Fig. 1), constituting 9.3% of the cohort. The most prevalent mutation was Ex19Del, observed in 3.5% of cases, followed by the L858R substitution at 2.9%, confirming the facts reported in the literature [12-15]. Other identified mutations included L861Q (0.9%), InsEx20 (0.7%), and G719X (0.7%). Notably, three instances of compound mutations were detected: G719X + S768I, L858R + InsEx20, and L861Q + G719X.

Concerning rare mutations, insertions in Exon 20 were identified in fewer cases than point mutations, but ranked next in frequency. Their lower identification can be attributed to the Real-Time PCR technique used, as insertions in Exon 20 are more readily identified through NGS (Next-Generation Sequencing) [12].

The majority of patients, totaling 391, yielded negative wild-type results.

Regarding patients with EGFR gene mutations, a salient aspect concerns the locations of metastasis occurrence. In the studied cohort, where available, EGFR-positive patients exhibited metastases in the brain, liver, and bones (e.g., one patient with the compound mutation L858R + InsEx20 showed brain metastasis, two with liver metastases— one with DelEx19, one with InsEx20— and two with bone metastases— one with Ex19Del, one with InsEx20). Notably, the InsEx20 mutation was identified in a patient with multiple metastases in the brain, liver,

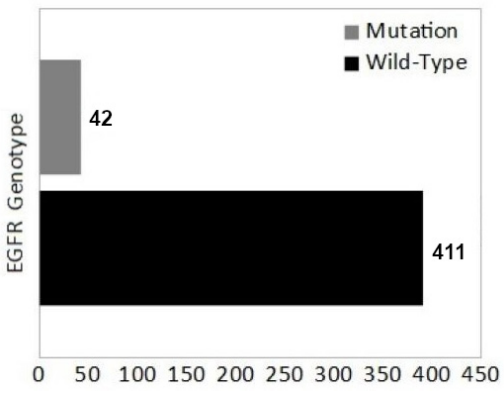

Figure 1. EGFR genotype among study patients.

bones, and adrenal gland. A South Korean study [16] analyzing a cohort of 1108 metastatic NSCLC patients (2015-2017) with 3% EGFR gene mutations, reported a frequent association of Exon 20 insertions with liver metastases. An Indian study observed a higher frequency of brain metastases in patients with common mutations compared to those positive for rare mutations (55.6% vs. 21.7%) [17].

Correlations between clinical-pathological characteristics and EGFR gene mutations

A gender-based comparison on EGFR gene mutations reveals a significantly higher prevalence among females (18.5%) compared to males (5.2%), aligning with existing literature indicating a higher frequency of EGFR gene mutations in women [13, 15, 18-21].

Of note, 37.5% of non-smokers exhibit EGFR gene mutations, whereas only 1.9% of active smokers and 8% of former smokers manifest such mutations (Table 3). Employing the chi-square test to assess the association between EGFR gene mutations and smoking status yielded a significant correlation ($\chi2=13.857$; df=2, p=0.001). EGFR gene mutations are notably more prevalent in non-smokers compared to smokers or former smokers, in accordance with literature [19-23]. A 2019 South Korean study reported a higher frequency of Exon 20 insertions among non-smokers [16]. Additionally, a 2022 Taiwan study suggests a correlation between smoking and the emergence of NSCLC forms associated with compound mutations in the EGFR gene [24].

The chi-square test results confirm a significant association between EGFR and exposure to a toxic environment ($\chi2=5.867$; df=1; p=0.015). Among non-exposed individuals, 33.3% exhibit EGFR mutations, while none of the exposed individuals show EGFR positivity. Caution is advised in endorsing this statement due to the limited data on exposure to a toxic environment, with only 6 individuals in the non-exposed group.

Patients with EGFR-negative status have a mean age of 65.11 years, while those with EGFR-positive status have an average age of 65.93 years. Utilizing the independent samples t-test reveals no statistically significant age difference between EGFR-positive and EGFR-negative individuals (p=0.595), but still, the result is consistent with findings from other studies in the literature [18, 19]. A statistically significant association (p<0.001) is observed between histopathological diagnosis and EGFR status. EGFR mutations were exclusively identified in patients with lung adenocarcinoma (38 of the 42 patients with EGFR mutations; histopathological diagnosis information is unavailable for the remaining 4 patients).

The larger number of tested patients diagnosed with adenocarcinoma corroborates the more frequent association of this NSCLC type with EGFR gene mutations [20, 21, 25]. This association may also be attributed to the specific recommendation for testing these mutations, particularly in patients with this histopathological diagnosis.

Association of EGFR-positive status with PD-L1 expression level and ALK rearrangements

PD-L1 exhibited positive results in 46.6% of the sampled patients, with an average expression level of 38.71%. ALK demonstrated positivity in 7.3% of the studied sample, with an average expression level of 51%. Employing the chi-square test to assess the relationship between PD-L1 and EGFR gene mutations revealed no significant association (p=0.877). Specifically, the proportion of individuals with positive EGFR did not significantly vary based on PD-L1 expression level. The chi-square test result also indicated no statistically significant association (p=0.212) between ALK and EGFR; the proportion of positive EGFR cases remained similar regardless of the ALK result (Table 4).

The mean expression levels of PD-L1 and ALK were compared between individuals with EGFR positive-status and those with EGFR negative-status in this study. Regarding PD-L1 expression levels among patients lacking EGFR gene mutations, a noticeable elevation was observed on average compared to patients with positive EGFR gene mutations (40.37% versus 26.13%). However, the results of the independent samples t-test indicated no significant differences between individuals with EGFR gene mutations and those with wild-type EGFR status,

Table 3. The variation of presence of EGFR gene mutations according to smoking status

			EGFR		Total
			Negative	Positive	
Smoking status	**Non-Smoker**	Frequency	5	3	8
		%	62.5%	37.5%	100.0%
	Active smoker	Frequency	53	1	54
		%	98.1%	1.9%	100.0%
	Ex-Smoker	Frequency	23	2	25
		%	92.0%	8.0%	100.0%
Total		Frequency	81	6	87
		%	93.1%	6.9%	100.0%

Table 4. The variation of presence of EGFR gene mutations according to PD-L1 expression and presence of ALK rearrangements

			EGFR		Total
			Negative	Positive	
PDL	**Negative**	Frequency	46	6	52
		%	88.5%	11.5%	100.0%
	Positive	Frequency	184	20	204
		%	90.2%	9.8%	100.0%
	Negative (+ immune cells)	Frequency	159	16	175
		%	90.9%	9.1%	100.0%
	Total	Frequency	389	42	431
		%	90.3%	9.7%	100.0%
ALK	**Negative**	Frequency	354	41	395
		%	89.6%	10.4%	100.0%
	Positive	Frequency	29	1	30
		%	96.7%	3.3%	100.0%
	Total	Frequency	383	42	425
		%	90.1%	9.9%	100.0%

concerning both PD-L1 expression level (p=0.075) and ALK expression level (t=0.609).

Investigating the association between PD-L1 expression levels and EGFR gene mutations is crucial, as literature suggests varying responses to potential immunotherapy in patients with these mutations. Recent studies indicate limited efficacy of targeted anti-PD-L1 treatment in EGFR gene mutation-positive patients. However, a 2020 Japanese study suggests that PD-L1 inhibitors are highly effective if the PD-L1 expression level exceeds 50% in EGFR mutation-positive patients [26]. International guidelines (NCCN, WHO) recommend anti-EGFR tyrosine kinase inhibitors as first-line treatment, reserving immunotherapy for cases of tumor progression.

Approximately 5% of NSCLC patients, mostly with adenocarcinoma, exhibit rearrangements in the ALK gene, typically showing resistance to anti-EGFR tyrosine kinase inhibitors [27]. The observed association in one patient in our study cohort of the L858R mutation and positive ALK may be attributed to tyrosine kinase inhibitors resistance. However, the lack of clinical data for this patient, coupled with the singular nature of this case, hinders definitive conclusions regarding the association of EGFR gene mutations with ALK positivity. Nevertheless, recent reports have outlined the simultaneous occurrence of these events in 0.1–2.4% of patients with NSCLC [28].

Treatment and evolution of patients with EGFR gene mutations

Among the 42 patients positive for EGFR gene mutations, 12 deceased, while 29 survived.

For EGFR-positive patients in the clinic under study, primary treatment predominantly involved Osimertinib (a third-generation TKI, recommended by NCCN guidelines). Exceptions included a stage IA adenocarcinoma patient, who, after complete tumor resection, required no further treatment, and a patient declining targeted therapy, opting for systemic treatment. Within 1 to 2 years of targeted treatment initiation, three EGFR-positive patients experienced tumor progression, likely indicative of third-generation TKI resistance.

A single patient, diagnosed with lung adenocarcinoma at age 70 and positive for the L861Q mutation in March 2022, sought retesting in October 2022 through a new biopsy. The lack of additional clinical data makes it inconclusive, whether retesting aimed to monitor resistance mutation emergence. Initial testing inability for PD-L1 and ALK markers due to tumor material depletion might have prompted the need for rebiopsy and retesting.

Three patients in the cohort transitioned from TKI treatment to immunotherapy or combined immunotherapy with chemotherapy due to tumor progression, without rebiopsy and retesting for EGFR gene mutations. The management of NSCLC patients and their retesting for resistance mutations may necessitate further investigation.

CONCLUSIONS

This study substantiated several findings reported in the literature. Notably, it confirmed the prevalence of EGFR gene mutations in patients diagnosed with lung adenocarcinoma, highlighting the identification of deletions in exon 19 (DelEx19) as the most frequent, followed by the L858R point mutation. Moreover, it emphasized the prevalence of EGFR gene mutations in female patients and non-smoker patients. Additionally, the present study elucidated an overall diminished expression level of PD-L1 in patients with EGFR gene mutations compared to Wild-Type counterparts. It further corroborated the

infrequency of ALK gene rearrangements in NSCLC patients, an occurrence even rarer when concomitant with EGFR gene mutations. Although existing data pertaining to the treatment and prognosis of EGFR-positive NSCLC patients are limited, they serve as a catalyst for future investigations into the resistance mechanisms to anti-EGFR TKIs in NSCLC patients in Romania, as well as the prevalence of EGFR gene mutations associated with this phenomenon.

ABBREVIATIONS

ALK – Anaplastic Lymphoma Kinase
EGFR – Epidermal Growth Factor Receptor
ESMO- European Society for Medical Oncology
EU – European Union
FFPET- Formalin-fixed paraffin-embedded tissue
HRP- Horseradish Peroxidase
NCCN- National Comprehensive Cancer Network®
NGS- Next-Generation Sequencing
NOS – Not Otherwise Specified
NSCC – Non-Small Cell Carcinoma
NSCLC – Non-Small Cell Lung Cancer
PD-(L)1- Programmed death (ligand) 1
TKIs – Tyrosine-Kinase Inhibitors
WHO- World Health Organization

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AUTHORS' CONTRIBUTION

All authors had equal contribution to this work.

All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

None to declare.

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