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EGFR MUTATIONAL STATUS IN SARDINIAN PATIENTS WITH LUNG ADENOCARCINOMA

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INTRODUCTION

Lung cancer is the most incident malignant neoplasia and the first cause of death for oncological disease throughout the world, with approximately 1,600,000 new cases and 1,370,000 deaths estimated in 2008[1]. Incidence rates in general population are closely related to the incidence of tobacco smoking, as the greatest part of lung cancer cases are linked to this single risk factor[2]. A certain decrease in the incidence of lung cancer in men was registered in the last decades in most Western countries, as a consequence of the multiple campaigns adopted against smoking. Nevertheless, incidence rates continuously increase in developing countries and in women, due to a progressively increasing adoption of smoking habits[3]. The world standardized incidence rates of lung cancer increased by 22% among females and decreased by 3% among males in the period 1985–2002[4]. Giving the current smoking trends, it is calculated that by 2030 lung cancer will affect both sexes equally[5].

Despite recent developments in all the scientific, diagnostic, clinical and surgical fields related to lung cancer research and management, mortality rates

remain high. The 5-year relative survival rate for lung cancer for the period of 1995 to 2001 was 15.7%, reflecting a steady but slow improvement from 12.5% from 1974 to 1976.2[6]. More recent studies estimate a 5-year survival rate of approximately 16% in the USA[7]. Several factors determine such high rates of mortality in patients with lung cancer. The most relavant are: a) insufficient campaigns against smoking, pollution and other risk factors for lung cancer b) lacking of effective screening strategies, c) subclinical evolution of early stage disease, d) delays on the diagnosis and clinical assessment of patients with suspicious signs and symptoms, e) insufficient comprehension of the pathophysiological mechanisms of the disease, and as a consequence f) lacking of effective treatment strategies, especially for patients with advanced stage disease.

Despite our knowledge of the pathophysiology of lung cancer is quite poor, a great amount of research was performed on this topic, especially in the last two decades, and some results were obtained and applied in clinical practice. One of the most relevant scientific conquers in recent times was the understanding of the role of the deregulation of the epidermal growth factor receptor (EGFR) in patients with non-small cell lung cancer (NSCLC). It was evidenced that EGFR was often overexpressed and aberrantly activated in NSCLC, and various activating mutations within the kinase domain of the EGFR gene were detected since 2004 in lung adenocarcinomas[8]. These tumors were found to be highly sensitive to the inhibitors of the EGFR-tyrosine kinase

(TKIs). TKIs were subsequently introduced in clinical practice, offering an additional therapeutic option in patients with lung adenocarcinomas. Unfortunately, the frequent arising of resistance to TKIs attenuated the initial enthusiasm related to the use of these agents[8]. Nevertheless, the discovering of EGFR mutations in patients with lung cancer remains of great importance for their clinical management and prognosis.

Several techniques were proposed and are actually in use for the detection of EGFR-mutations. The current continuous technological progression leads gradually to a major refinement and improvement of the existing techniques, and to the birth of new methods and approaches for the detection of EGFR gene mutations. However, the quality of the specimen available for analysis remains a factor that can profoundly influence EGFR mutational studies. This factor is extremely important, especially in non-surgical patients, considering the intrinsic technical difficulties of lung biopsy methods, and given the anatomy of the lungs and the possibility for complications that may be severe in some instances.

EGFR mutation studies in patients with lung cancer have been widely published in recent years, delineating the mutational status of the gene in several populations throughout the world. The aim of this doctoral thesis was to investigate the EGFR mutational status in North Sardinian patients with lung adenocarcinomas.

LUNG CANCER EPIDEMIOLOGY IN NORTH SARDINIA, ITALY

A study on the incidence and mortality rates and trends of lung cancer in North Sardinia in the period 1992 – 2010 has been recently published by the Author of the present doctoral thesis, in collaboration with the Service of Epidemiology of the health agency of Sassari and the Cancer Genetics Unit of the National Research Council (CNR)[3]. The epidemiological data presented in this study were obtained from the "Registry of the tumors of the Province of Sassari". This registry was created in 1992 by the local health agency for the epidemiological surveillance of tumors in the province. In 1999 it became part of a wider web of tumor registries, coordinated today by the Italian Association for Tumor Registries (Associazione Italiana Registri Tumori, AIRTUM). The association coordinates 34 registries in the country, collects and publishes data, and collaborates with international organizations in the field.

Every registry collects data on tumoral diseases affecting inhabitants in the territory of jurisdiction through the local hospitals and health care services,

as with other registries (e.g., death registries). Demographic, clinical, pathological and prognostic data are collected for each case of cancer and are registered in a digital database. This database was the data source for the study of Paliogiannis et al[3].

In this study the demographic characteristics of the patients affected by lung cancer were collected. Crude incidence and mortality rates per 100,000 inhabitants per year were calculated, as were standardized rates adjusted for European age-population standards. A comparison between incidence and mortality in the province of Sassari and those in other Italian provinces was performed. Additionally, the cumulative risk of developing the disease and of dying between zero and 74 years of age was estimated. The age class distribution and time trends of incidence, mortality, mean age of disease onset and death, as well as histology were evaluated. Finally, relative 5-year survival was calculated.

The overall number of cases of lung cancer registered in the period under investigation was 4,325. Diagnosis was obtained by histological or cytological reports in 3,178 cases (73.5%) and using other information sources (clinical reports, radiological referrals, and death certifications) in 1129 cases (26.1%). The modality of diagnosis was not known in 18 cases (0.4%). Among the 4,284 individuals registered, 3,554 were males and 771 females, with a male-to-female ratio of 4.6:1. The mean age was 68.1 years for males and 67 years for females.

The cumulative risk of developing the disease was 6.13% for males and 1.11% for females.

As regards the anatomical distribution of the tumors 568 (13.1%) were found in the tracheo-bronchial tree, 1493 (34.5%) in the upper lobes, 231 (5.3%) in the right middle lobe, 781(18.1%) in the inferior lobes and 272 (6.3%) in more than one lobe, while in 980 (22.7%) cases the anatomical localization was not known. Among the 3,178 tumors that had histological or cytological diagnosis, 1330 (41.9%) were adenocarcinomas, 845 (26.6%) were squamous cell carcinomas, 310 (9.8%) were small cell cancers, 88 (2.8%) were large cell cancers and 126 (4%) were other histotypes, while in the remaining 479 (15%) cases the exact histologic type was not specified. Figure 1 depicts the trend of the principal histotype rates in the years under investigation.

The crude incidence of lung malignancies in the period under investigation was 86.5/100,000 for men and 18.1/100,000 for women. Standardized incidence rates were 73.1/100,000 for males and 13.5/100,000 for females.

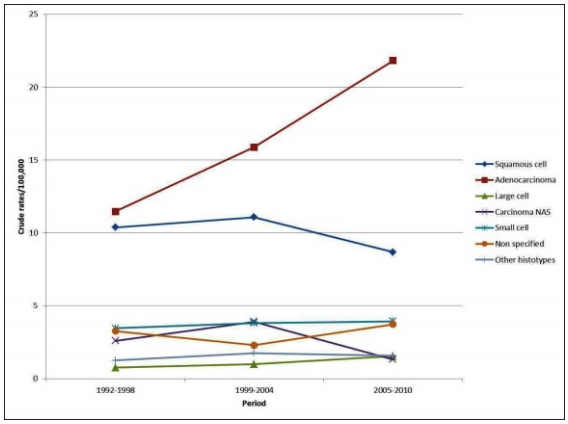


Figure 1. Trends in histological subtypes of lung cancer in North Sardinia, 1992-2010[3].

Peak incidence occurred at 75-79 years for both males and females. Incidence rates were also calculated for the following three time periods: 1992–1998, 1999–2004 and 2005–2010 (Figure 2). There was a progressive decrease in incidence rates in males, from 78.7/100,000 in the first period, to 72.3/100,000 in the second period and 67.3/100,000 in the last period. The corresponding figures for females were 10.1/100,000, 13.5/100,000 and 17.4/100,000, respectively. A constant increase in incidence occurred between 1992 and 2010 in women. Analysis of the trend of mean age at disease onset for the same periods of time did not reveal any relevant changes. Table 1 shows the comparison of the incidence and mortality in the province of Sassari with those in other Italian provinces.

There were 3347 deaths in the period under investigation (2751 males and 596 females). Crude overall mortality was 67/100,000 for males and 14/100,000 for females. Mean age at death was 69.4 years in males and 69.2 years in females. Standardized mortality rates were 55.7/100,000 for males and 9.9/100,000 for females.

The cumulative risk of death was 4.47% for males and 0.78% for females. There was a relevant increase in mortality rates after the fifth decade of life. Figure 2 shows the time trend of mortality between 1992 and 2010: a significant increase in mortality in both sexes was registered. Finally, relative survival at 5 years from diagnosis was 10% (8.8% for males and 14.9% for females).

Province	Incidence (/100,000 per year)		Mortality (/100,000 per year)	
	Males	**Females**	**Males**	**Females**
Alto Adige	59	16.2	51.9	12.7
Biella	94	16.3	80.1	14
Ferrara	96.8	18.7	81.7	16.2
Firenze	77.4	17.1	65.5	12.7
Friuli V.G.	78.8	19.2	71.5	16
Genova	97.9	16.6	80.1	12.6
Macerata	64	11.9	57	8
Modena	82.9	18.6	72.9	15.4
Napoli	94.8	13	83	10.6
Parma	80.8	20	65.2	14.9
Ragusa	65	8.4	56.6	7.3
Reggio Emilia	76.9	19	69.9	14.5
Romagna	87.7	20.3	71.5	14.5
Salerno	74.3	8.8	66.1	7.6
Sassari	**73.1**	**13.5**	**55.7**	**9.9**
Torino	87.9	18.2	75.7	14.2
Trento	68	11.8	66.6	10.7
Umbria	66.7	14.2	55.4	9.8
Varese	84.1	14.5	81.1	12
Veneto	95.1	21.1	85.7	15.5

Table 1. Comparison with incidence and mortality rates of other Italian provinces, 1992-2010[3].

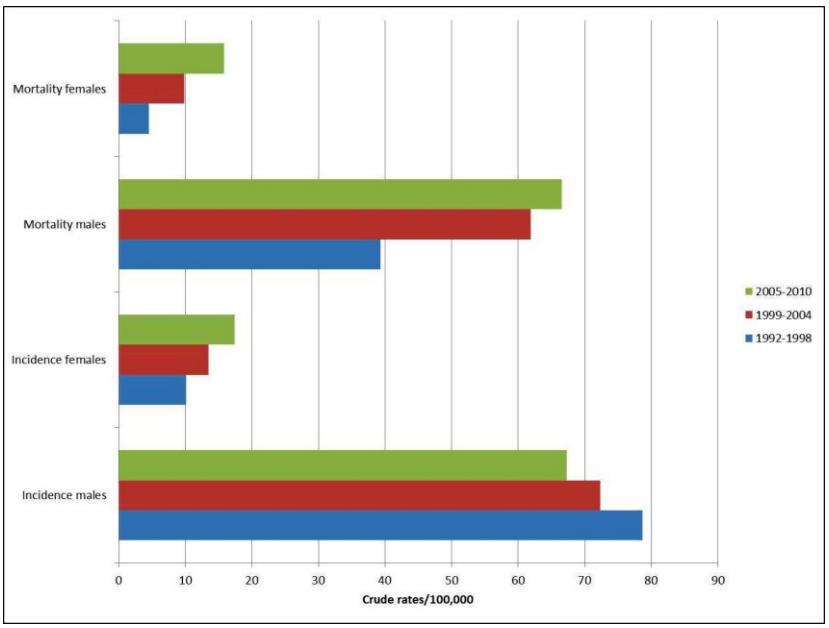


Figure 2. Incidence and mortality rates trends of lung cancer in North Sardinia, 1992-2010[3].

Standardized incidence rates in the province of Sassari were similar to those estimated by AIRTUM for northern Italian regions. Comparisons of the incidence rates with those of other Italian provinces place the province of Sassari in the middle between those with low incidence rates, such as Alto Adige and Macerata, and those with higher incidences like Genova, Ferrara and Veneto.

Concerning histology, a prevalence of adenocarcinomas over squamous cell carcinomas and other subtypes was observed in the area. Furthermore, a decline in incidence of squamous cell carcinomas was evidenced, as opposed to other histological types which presented a slight increase in incidence rates between 1992 and 2010. A remarkable shift in world lung cancer incidence rates by histologic subtype occurred in the last decades. Squamous cell carcinoma was the most frequently observed histotype in the initial epidemiological reports

since the 80's when it was superseded by adenocarcinoma[5,9]. The causes of such shifting are not clear and several hypotheses have been proposed, concerning mainly factors linked to tobacco smoking: changes in the characteristics of cigarettes, increased puff volume, increased nitrate levels and, consequently, higher nitrosamine levels[10,11].

Considering the distribution of the disease in relation to age in North Sardinian patients, less than 5% of the cases occurred in individuals ≤44 years, while more than 75% occurred after the sixth decade of life. This reflects the modalities of exposure to risk factors like smoking and pollution and the long latency from exposure to disease. Incidence rates increased with aging in both sexes, reaching peak values in individuals ≥80 years. This distribution pattern is similar to those reported for the world population[12].

The time trends analysis performed in the study showed a steady increase in incidence of lung cancer in females in Sassari province in the period under investigation. Conversely, a slight reduction in incidence rates was observed in males. These trends are common to other national and international geographical areas, and may reflect the increasing diffusion of tobacco smoking in women as opposed to the reduction of smoking incidence in men. The world standardized incidence rates of lung cancer increased by 22% among females and decreased by 3% among males in the period 1985-2002[13]. Giving the current smoking trends, it is calculated that by 2030 lung cancer will affect both sexes equally[5].

The role of screening programs in patients with risk factors for lung cancer is still a matter of debate. Some studies, especially those performed using standard chest X-ray, did not reveal any impact of the screening program on survival. More recent contributions, with a longer follow up time available and better technologies employed, showed a relevant diminishing in mortality rates in enrolled patients, and posed the question of the utility of screening strategies in lung cancer prevention[14]. Nevertheless, several aspects remain to be addressed before the introduction and diffusion of screening campaigns in clinical practice. To date no screening programs for lung cancer are active in North Sardinia, as opposed to numerous smoking control campaigns.

Concerning mortality, 3,347 (2,751 males and 596 females) deaths occurred in the 18 years we studied. Standardized mortality rates were considerably inferior in women. Considering the age-class mortality trend, a natural increase in relation to age was observed in both sexes, with peaks after the eighth decade of life and with a slight increase between 1992 and 2010. Standardized mortality rates were increased in Sassari province in the years under investigation, as opposed to global national figures which evidenced a steady decrement of mortality rates in males in the last 2 decades (-2.2% per year) and a continuous increment in females (+1% per year) [15].

Finally, relative survival at 5 years from diagnosis was low in both sexes (10%), but in accordance with percentages published for other developed countries and for the entire country[16].

Several factors impact in such a low survival in patients with lung cancer such as lack of effective screening programs, non-specific clinical manifestations, delays in diagnosis, high percentage of advanced stages at diagnosis, smoking related comorbidities, ineffectiveness of current therapeutic strategies, and others. The relative 5 years survival was better in women, but the gap observed between sexes seems destined to diminish in the future as a consequence of the steadily increasing smoking incidences in women[5,9].

Concluding, the data of the study previously performed by the Author of the present thesis in collaboration with the institutions mentioned above, showed an increasing trend in incidence of lung cancer in women in North Sardinia in the last decades. Conversely, a reduction of incidence rates was observed in males. Furthermore, a slightly increasing trend in mortality rates was observed in both sexes, suggesting the need to enhance smoking control strategies, consider adoption of effective surveillance policies and to improve diagnosis and treatment methods.

EPIDERMAL GROWTH FACTOR RECEPTOR: PHYSIOLOGY AND PATHOPHYSIOLOGY

The epidermal growth factor receptor (ErbB) family is a member of the receptor thyrosin kinases (TKs) superfamily, including four different receptors: EGFR/ERBB1/HER1, ERBB2/HER2, ERBB3/HER3, and HER4[17,18]. These receptors are transmembrane glycoproteins, with a lingand-binding domain in the external surface of the cell membrane, and domains mediating signal transduction located in the cytoplasm. The receptors exist as monomers in their non-active state. Following ligand binding, the receptor undergoes conformational changes, and can homodimerize or heterodimerize with another receptor of the same family; this causes the transphosphorylation of key thyrosine residues in the activation loop of TK domains, through the transfer of phosphates from adenosine triphosphate (ATP)[19]. These events promote the activation of several intracellular cascades, which mediate the transduction of the signal through several downstreaming signaling actions[20]. All family members possess an intrinsic TK activity, except ERBB3.

The principal effects resulting by the activation of these receptors consist principally in modifications of gene expression, changes in the cytoskeleton, activation of cell adhesion mechanisms, proliferation and survival. The receptors are physiologically expressed in numerous epithelial, mesenchyme and nervous tissues[18,21]. The role of EGFR receptor family has been elucidated through studies performed on EGFR-gene knock-out mice, which presented high mortality rates due to placenta and lung alterations[21].

The EGFR is a 170-kDA protein that represents the first member of Erb receptors family, as it was the first to be discovered on the membrane of epidermoid cells. Similarly to the other receptors of this family, it is characterized by a three parts structure: an extra-membrane domain for the ligand, a single helical transmembrane domain, and a cytoplasmatic domain (Figure 3)[22]. The thyrosin-kinase domain represents approximately the 50% of the cytoplasmatic compound of the receptor, and it is composed by 38 extramembraneous and 225 carboxi-terminal aminoacids[23]. The transphosphorylation of this domain leads to signal transduction and, consequently, to cell proliferation, inhibition of apoptosis, angiogenesis and other biological effects[24,25].

Increased expression levels of EGFR have been described in several tumors (breast, prostate, gastric, colorectal) and in approximately 80% of NSCLC[26]. Furthermore, EGFR mutations can involve all the three mentioned above compounds of the receptor.

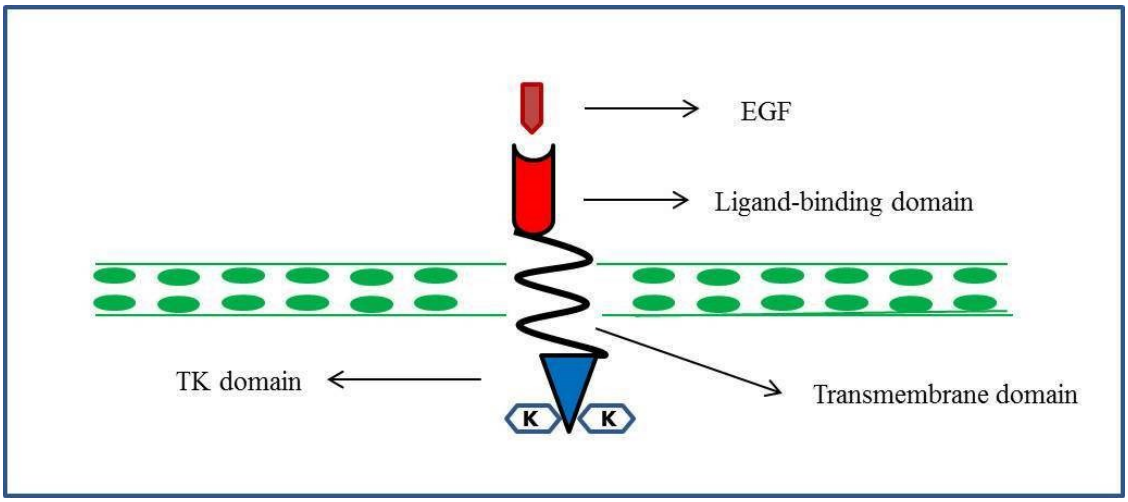


Figure 3. Scheme depicting the composition and cell location of EGFR.

Mutations involving the extra-membranous domain are generally delections, defined EGFR vI, EGFR vII or EGFR vIII, on the basis of the site and length of the deletion. Those mutations have been described in 5% of squamous lung carcinomas, but not in other histological NSCLC subtypes[27-29]. Mutations of the plasmatic extra-membrane aminoacid component are generally missense (V689M e L703F), and are involved in the receptor's oligodimerizzation process[23]. Finally, mutations of the thyrosin-kinase domain are generally aminoacid duplications in the kinasic region, which lead to a constitutive activation of the intracellular proliferative signals.

These later mutations are involved in mechanisms which lead to sensibility to TKIs, in patients with NSCLC, because they involve the site of action of the ATP, which is the same site of action of TKIs[30,31]. For this reason, these mutations must be considered as predictors of a clinical benefit for the patients, as they can be treated with targeted TKIs therapies.

THE ROLE OF EGFR MUTATION ANALYSIS IN THE MANAGEMENT OF NSCLC

Since its discovering in 1986, EGFR appeared to be extremely important in the oncogenesis of several tumors, because of its effects on cell proliferation, angiogenesis and apoptosis[24,25]. Subsequently, EGFR was found to be frequently overexpressed or aberrantly activated in patients with NSCLC[32]. The existence of activating mutations in lung cancer was firstly described in 2004 and widely confirmed thereafter[33]. The discovery of mutations of the thyrosin kinase domain of EGFR gene is of great importance, especially in patients with adenocarcinomas, because large part of the tumors presenting such mutations are sensitive to TKI agents[33].

EGFR mutations occur in the thyrosin kinase domain of the gene, which comprises exons 18 – 24. In particular, the mutations mainly involve exons 18 – 21. Up to 90% of these mutations are delections in exon 19 and point mutations in exon 21 (Figure 4)[34]. Mutations in exons 18 and 20 are rarer. According to

numerous recent reports the distribution of EGFR mutations in the first four exons of the TK domain of the gene is as follows:

- exon 18: 4-6%
- exon 19: 45-50%
- exon 20: 4-6%
- exon 21: 40-45%

Mutations in exons 19 e 21 lead to activation of the receptor, due to constitutive autophosphorylation[19]. As regards the signal pathways, the signal transducer and activator of transcription (STAT) and Akt pathways seem to be involved mainly, inducing cell survival; the mitogen-activated protein kinase (MAPK) pathway, that induces proliferation, appears to be less involved[35].

Different mutations present different oncogenic potential: deletions in exon 19 are associated with higher malignant behavior in comparison to exon 21-point mutations[8]. Globally, patients with EGFR mutation have a better prognosis in comparison to those without mutations, and this leaded several Authors to hypothesize that NSCLCs with EGFR mutations represent a distinct biological entity[36,37].

EGFR mutation prevalence varies among different populations and subsets of patients. The prevalence of these mutations varies from 20 to 40% in Asiatic populations, from 5 to 20% in whites, and are rarer in blacks[38]. Additionally, the incidence of exon 19 mutations seems to be higher in European populations in comparison to Asiatics.

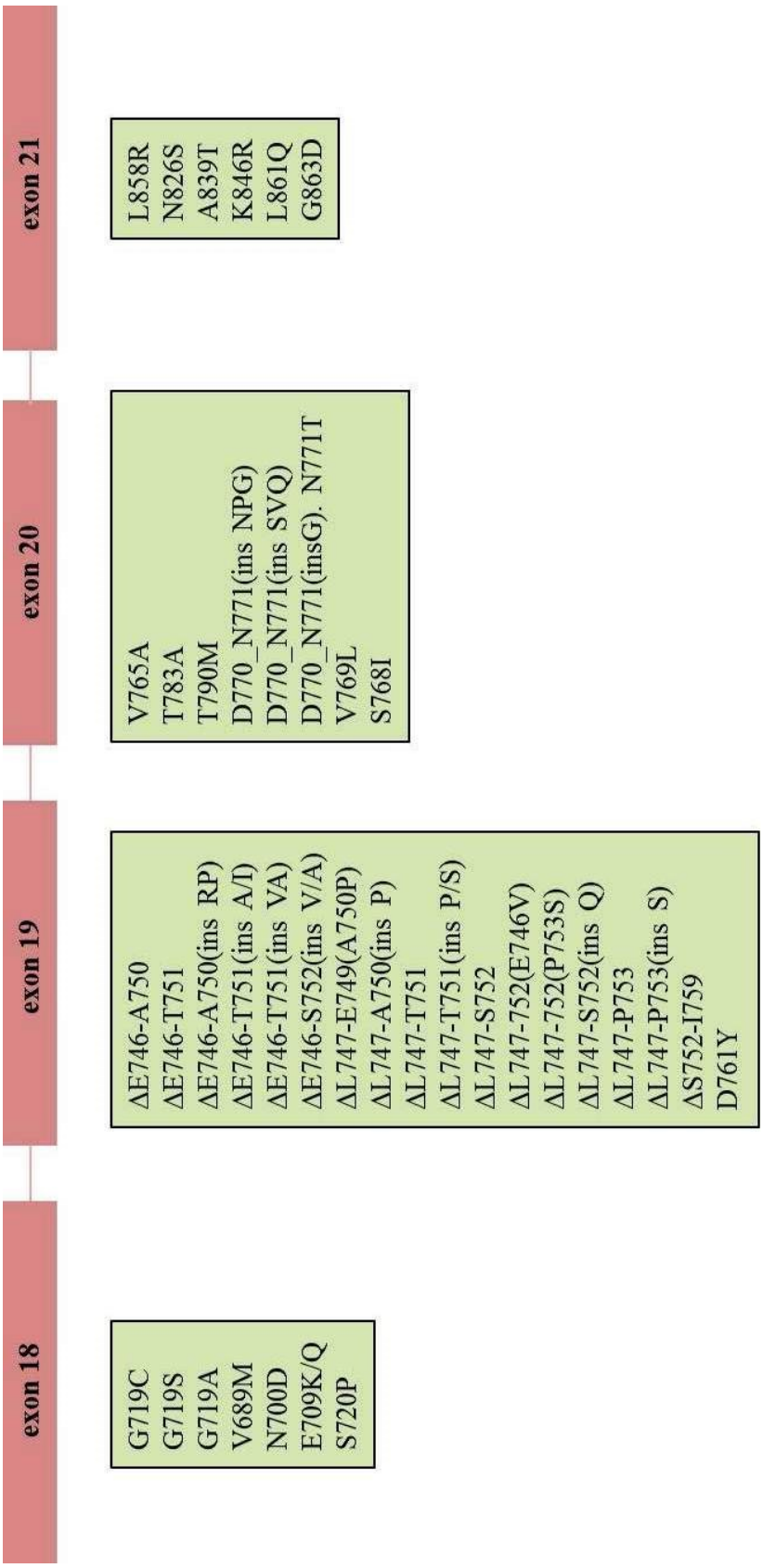


Figure 4. Distribution of main EGFR mutations in exons 18-21.

Females and never smokers present significantly higher proportions in comparison to males and active or former smokers respectively[39,40]. Furthermore, adenocarcinoma is the histotype that most frequently presents EGFR mutations; large cell carcinomas and squamous carcinomas are rarely involved[40]. In all histotypes, EGFR and Kras mutations are mutually exclusive.

As we mentioned before, EGFR mutations in exons 19 and 21, represent approximately the 85-90% of the overall, and have been largely demonstrated to offer prognostic advantages in patients treated with TKIs[33]. Less is known for the so called "non-common" EGFR mutations, given their low incidence. At the contrary, it is known that some EGFR mutations are responsible for the development of resistance to TKIs; the most frequent one is an insertional mutation in exon 20 (T790M)[41].

Another mechanism of EGFR activation is represented by the increased number of gene copies. It has been reported that an increase in the EGFR copy number is more predictive of survival than EGFR mutations after TKI treatment[42]. Nevertheless, some Authors include in the definition of increased gene copy number both amplification and high polysomy; it is not yet clear if these events have the same biological impact on EGFR gene activation.

Others found high numbers of novel mutational variants in their studies, but it is possible that some of these were artefactual rather than real mutations[43].

Japanese Authors reported that the increasing of EGFR copy number was not predictive of TKIs effectiveness[44,45]. Other Asiatic Authors reported higher

response rates in TKI therapies in patients with tumors presenting both EGFR mutations and high gene copy number in univariate analysis; in multivariate analysis only EGFR mutations were confirmed to produce this effect[46]. Generally, it has been proven that lung tumors with EGFR mutations tend to present also gene amplification, but it is possible that ethnic and racial factors may impact the role of EGFR alterations and their oncological significance. These speculations should be better addressed in future prospective well-designed clinical trials.

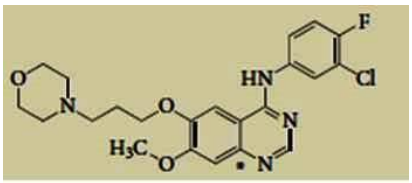
THYROSIN KINASE INHIBITORS

The first anti-EGFR therapies were developed in the 1990s. The agents used at the time were directed against the wild-type receptor, because it was found to be overexpressed in several types of epithelial tumors[33]. These agents included the small TKI molecules gefitinib (Iressa®; AstraZeneca) and erlotinib (Tarceva®; Genentech/OSI Pharmaceuticals).

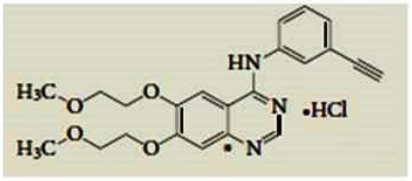
Gefinitib and erlotinib are currently the TKIs approved for clinical use in patients with NSCLC. These molecules make part of a larger family, which comprises numerous agents: axitinib, lapatinib, imatinib, sunitinib, vatalanib, semaxanib and others. Some of these agents are currently under investigation in different phases of NSCLC treatment. The chemical formulations of the most relevant TKIs employed for clinical and experimental purposes are depicted in Figure 5.

The mechanism of action of TKIs is based on their ability, given their small dimensions, to be selectively inserted in the kinasic site of the cytoplasmatic domain of EGFR and to reversely block phosphorylation. In other

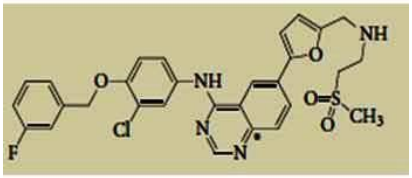
words, TKIs are competitors with ATP for its site of action in the kinasic domain[47]. TKIs which act through a covalent binding to the action site are currently under investigation for clinical use.



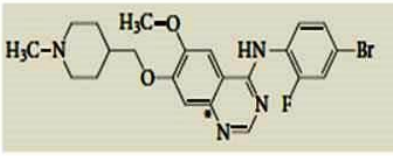
Gefitinib



Erlotinib



Lapatinib



Vandetanib

Figure 5. Chemical formulations of principal TKIs involved in current clinical and experimental practice.

The use of gefitinib in patients with advanced NSCLC in the 1990s, leaded to the observation that only about 10% of the cases presented

radiologically evident responses[48,49]. The main demographic and clinical characteristics of responders were found to be Asian ethnicity, female sex, never smoking history, and adenocarcinoma histology[50,51]. EGFR thyrosin kinase domain mutations were discovered in 2004 and it was observed that NSCLC patients with these mutations presented the same demographic and clinical characteristics mentioned above[33]. This leaded to the hypothesis that responders to erlotinib were those with EGFR thyrosin kinase domain mutation. This hypothesis was subsequently confirmed, albeit some initial difficulties because the early studies were often inconclusive or contradictory, given their retrospective design and several methodological and technical limitations.

Most of the clinical trials which definitely confirmed the association between EGFR mutations and sensitivity to TKIs were published in the late 2000s. These studies were performed in Europe, United States and Asia, testing two different TKIs: erlotinib and gefitinib. Results were generally evaluated using the following parameters: radiographic response rates (RRRs), progression free survival (PFS), and time to progression (TTP). Figures calculated were 55-91% for RRRs, and from 7.7 to 13.3 months for PFS and TTP[33]. The corresponding figures for unselected patients with NSCLC treated with TKIs were 8-9% and 2.2 to 3 months respectivelly[52,53]. Finally, more recent well designed studies showed that TKIs are superior to conventional chemotherapy, when used as an initial treatment for EGFR-mutant NSCLC[54]. Additionally, these studies evidenced lower response rates to TKIs in the second line, in

comparison to TKIs used in the first line[54]. Nevertheless, further studies are necessary to confirm these findings.

Another interesting finding was the fact that a variable amount of patients (ranging from 1% to 20%) without EGFR-mutant tumors, presented a response to TKI treatment[55,56]. This finding can be explained by technical limitations of current technologies in the detection of EGFR activating mutations. Furthermore, it is possible that genetic alterations other than EGFR mutations may activate the EGFR signaling pathway, but this is currently a matter of further research.

As we mentioned before, increased EGFR copy number (polysomy and gene amplification) is often associated to NSCLC, with or without EGFR mutations. Increased EGFR copy number has been associated to better prognosis in some retrospective studies, but this finding was not confirmed in more recent prospective studies and must be better assessed in the future[42].

Unfortunately, a quote of NSCC is resistant to TKIs, despite the presence of an EGFR activating mutation. It has been estimated that this quote represents approximately the 25% of EGFR mutant cases[33]. Resistance to TKIs can be primary or secondary to TKI treatment. Primary resistance may be due to drug-resistant EGFR mutations or due to other genomic alterations that occur simultaneously to EGFR mutations. Drug-resistant mutations have been found in all exons involved in EGFR mutagenesis; the most relevant appears to be T790M deletion in exon 20[41,57]. This mutation accounts for about a half of all

the mutations of exon 20. Genomic alterations involving different genes, like mutations of PIK3CA and PTEN loss of expression, have been described to be linked with partial or total resistance to TKIs[58,59]. On the other hand, second site mutations, MET amplification, and other molecular mechanisms have been found to impact the arousal of acquired or secondary resistance to TKIs[33].

EGFR MUTATIONAL STATUS IN SARDINIAN PATIENTS WITH LUNG ADENOCARCINOMA

Assessment of the EGFR mutational status has become, as we mentioned before, a crucial step in the molecular classification of the patients toward the treatment decision, and makes currently part of the standard clinical management of patients with lung adenocarcinoma. The aim of this doctoral thesis was to investigate the EGFR mutational status in North Sardinian patients with lung adenocarcinomas.

Materials and Methods

Samples

Five hundred and fifteen patients with histologically-proven diagnosis of NSCLC and regularly participating to the follow-up programs at the Institutions across Sardinia island were included into the study. To avoid any bias, NSCLC patients were consecutively collected from September 2010 to May 2013; they were included regardless of age at diagnosis and disease characteristics.

Sardinian origin was ascertained in all cases; for all patients, place of birth of their parents was assessed in order to assign their geographical origin within the island. Clinical and pathological features for the assessment of the disease stage at diagnosis, as well as of the onset age and tumour anatomical location were confirmed by medical records and/or pathology reports. Formalin-fixed paraffin embedded tissue samples from NSCLC patients were obtained from the archives of the Institutes and Services of Pathology participating to the study. Tissue samples were evaluated for the content of neoplastic cells by light microscopy.

All patients were informed about the aims of this study and, before the tissue sample was collected, gave a written informed consent. The study was reviewed and approved by the ethical review board of the University of Sassari.

Mutation analysis

All tumour tissues were collected and processed at the laboratory of the Institute of Biomolecular Chemistry of Sassari; genomic DNA was isolated from tissue sections using a standard protocol and DNA quality assessed for each specimen. In particular, paraffin was removed from formalin-fixed paraffin-embedded (FFPE) samples by treatment with Bio-Clear (Bio-optica, Milan, Italy) and DNA was purified using the QIAamp DNA FFPE Tissue kit (QIAGEN Inc., Valencia, CA, USA).

The coding sequence and splice junctions of exons 19 and 21 (for all cases), as well as exon 18 for a large fraction of the patients (collection -

incompleteness was due to the low amount of available tumour tissue samples) in EGFR gene were screened for mutations by direct automated sequencing. Polymerase chain reaction (PCR) was performed on 25-50 ng of isolated genomic DNA in a 9700 Thermal cycler (Applied Biosystems, Foster City, CA, USA); all PCR-amplified products were directly sequenced using an automated fluorescence-based cycle sequencer (ABIPRISM 3100, Applied Bio-systems, Foster City, CA), as previously described by our group. Primer sequences and protocols for PCR-based assays were designed and optimized in our laboratory; they will be available upon request.

Statistical analysis

A descriptive analysis for qualitative and quantitative variables was carried out, using proportions and means (SDs), respectively. Furthermore, it was performed a correlation analysis between proportion of neoplastic cells and clinical variables and with the mutations found. Statistical significance was set up to a level ≤ 0.05. All the analysis were carried out using statistical software (STATA 12®, StataCorp LP, Texas, USA).

Results

Among the 515 cases examined 357 (69.3%) were males and 158 (30.7%) females. The mean age was 64.9 years (SD: 10.1). Three-hundred eighty-two

patients (84.5%) were active tobacco smokers or have had a history of smoking, while the remaining 133 (15.5%) were never smokers (Figure 6).

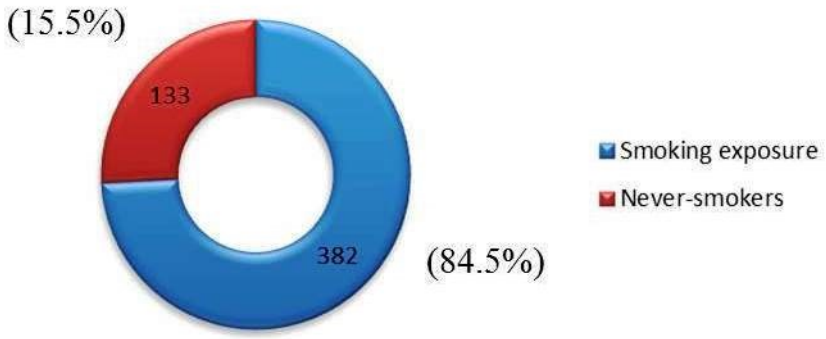


Figure 6. Distribution of cases by smoking habits.

Four-hundred fifty-six (88,5%) specimens were obtained by primitive lung lesions, and 59 (11.5%) by metastatic lesions (Figure 7). The anatomical distribution of metastatic lesions submitted to biopsy was as follows: liver 15 (25.4), lymph nodes 15 (25.4), bone 12 (20.3%), central nervous system 7 (11.9%), pleura 4 (6.8%), skin 2 (3.4%) and other tissues 4 (6.8%) (Figure 8).

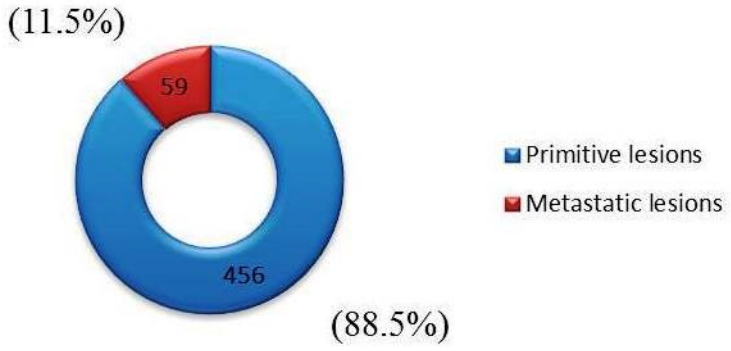


Figure 7. Distribution of cases by specimen origin.

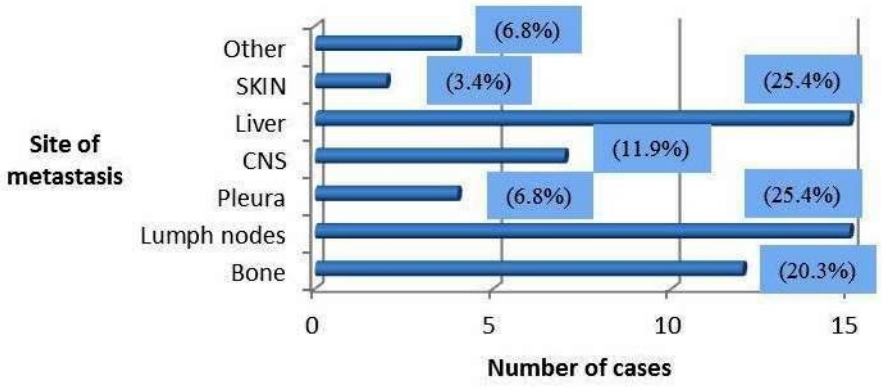


Figure 8. Distribution of metastatic lesions by anatomical site.

Four-hundred twenty-nine (83.3%) tissue samples were obtained by biopsy (transcutaneous or endoscopic), while 86 (16.7%) samples were obtained from surgical specimens (Figure 9).

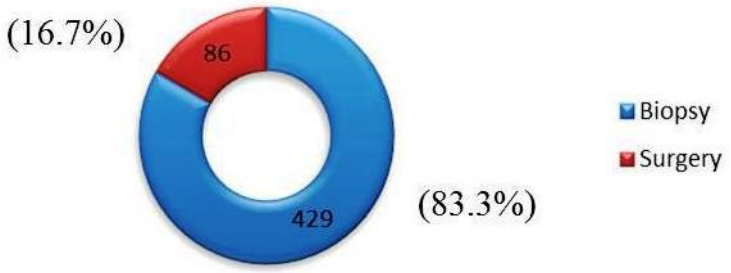


Figure 9. Distribution of cases by specimen retrieval modality.

The total number of EGFR mutations found was 59 (11.5%). EGFR-exon 18, EGFR exon-29 and EGFR exon-21 mutation proportions were 1 (1,7%), 30 (51%) and 28 (47.3%) respectively. The age – distribution of these mutations evidenced a single mutant case among the 6 patients with less than 40 years of

age examined (16.7%). The greatest number of mutations (18) was detected in patients in the sixth decade of life, but this group was the one with the higher number of cases examined (178). Considering proportions, EGFR mutations were discovered in approximately 10% of the cases examined in all age-groups higher than 50 years, while in patients aged between 40 and 50 years the proportion of mutations found was 21% (Figure 10).

The global number of EGFR mutations was significantly superior in women than in men, due to a consistently higher incidence of EGFR exon-19 mutation in females (Table 2).

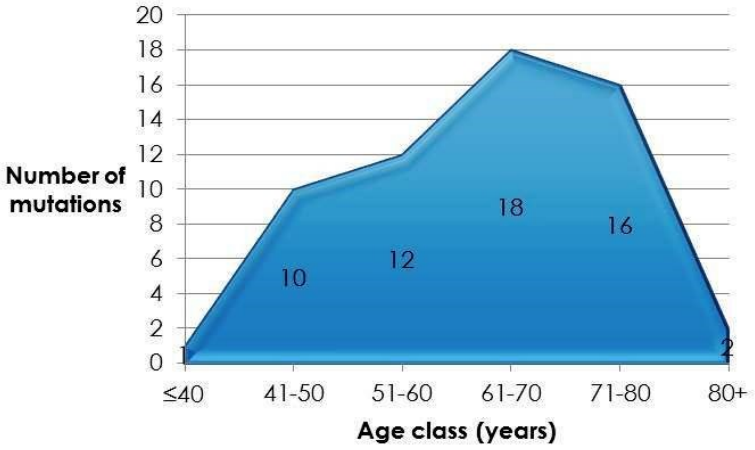


Figure 10. Age-class distribution of the EGFR mutations found.

Variables	Male	Female	p-value
EGFR status, n (%)	24 (6.7)	35 (22.2)	<0.001
EGFR-exon18 mutations, n (%)	0 (0.0)	1 (0.95)	0.15
EGFR-exon19 mutations, n (%)	12 (3.4)	18 (11.4)	<0.001
EGFR-exon21 mutations, n (%)	12 (3.4)	16 (10.1)	0.002

Table 2. Sex distribution of the EGFR mutations found.

As regards the smoking status, EGFR mutations found to be significantly more common in never-smokers, especially those of exons 19 and 21 (Table 3).

Variables	**Smoking exposure n= 382**	**No smoking exposure n= 70**	**p-value**
EGFR status, n (%)	22 (5.8)	37 (52.9)	<0.001
EGFR-exon18 mutations, n (%)	0 (0)	1 (1.4)	0.019
EGFR-exon19 mutations, n (%)	12 (3.1)	18 (25.7)	<0.001
EGFR-exon21 mutations, n (%)	10 (2.6)	18 (25.7)	<0.001

Table 3. Distribution of the EGFR mutations found in relation to the smoking status of the patients.

Finally, the distribution of the mutations among primitive and metastatic tissues evidenced no statistically significant differences in the proportions of EGFR mutations detected on primitive lung adenocarcinomas (51 mutations, 11.2%) and those found in metastatic samples (8 mutations, 13.5%).

The mean percentage of neoplastic cells in the samples employed for mutation analysis was 52.5% (SD: 9.8, range 20-90). The distribution of the percentages of neoplastic cells in the specimens is depicted in Figure 11; in more than 75% of the cases examined the percentage of malignant cells in the tissue sample was between 40%-60%, while in less of 1% of the cases the percentage was inferior to 20% or superior to 90%.

A statistically significant difference in the percentages of malignant cells was evidenced between samples with more than 50% of neoplastic cells (450 cases – 57 mutations) and those with less than 50% of neoplastic cells (65 cases – 2 mutations).

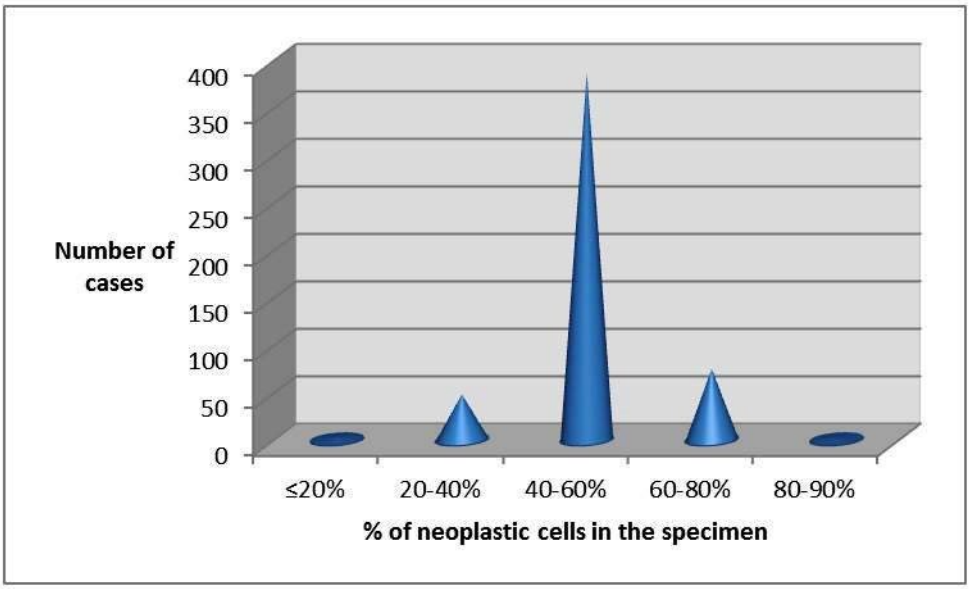


Figure 11. Distribution of cases by percentage of neoplastic cells in the specimen.

Furthermore, a statistically significant difference in the percentages of malignant cells was evidenced between samples obtained by biopsy and those obtained by surgery, with biopsy specimens containing slightly higher percentages. Conversely, no statistical differences were detected between primitive and metastatic tissue samples. Moreover, no statistically significant differences were found analyzing the various types of mutations in relation to the percentage of neoplastic cells in the specimens.

Discussion

Epidermal growth factor receptor mutations were found in 11.5% of Sardinian patients with lung adenocarcinoma. This percentage is similar to those reported in literature for other Caucasian populations[38]. Moreover, this percentage is also similar to those reported in recent years for other Italian areas.

Marchetti et al. in 2005 found 39 EGFR mutations among 375 patients with lung adenocarcinoma (10%), while Stella et al. reported in a more recent publication 13 (9.7%) EGFR mutations among 134 lung adenocarcinomas[43,59].

In our study EGFR mutations were found to be significantly more frequent in females and non-smokers than in males and former or active smokers. This finding has been extensively reported in numerous previous publications from different areas of the globe[39,40,50,51]. Furthermore, it has been advocated in the past that EGFR mutations involve more frequently patients with bronchioloalveolar (BAC) adenocarcinomas of the lung[39]. This finding was not confirmed in more recent publications; in our study three cases of pure BAC were registered, but none of them presented EGFR mutations. Generally, patients with this particular histotype have a better prognosis and better response rates to TKIs.

As regards the origin of the tissue specimen to use for mutation analysis, we did not found statistically significant differences in the percentages of EGFR mutations detected between samples obtained by biopsy and those obtained by surgery. Furthermore, no statistical differences in the percentages of mutations were found between tissue samples obtained from the primary tumor and those obtained from distant metastatic lesions, developed either through a lymphatic or hematogenous diffusion. This a very important aspect for the clinical management of patients with lung cancer, because of the invasiveness of surgical methods and the necessity to perform mutation analysis in small tissue

samples, obtained by biopsy methods. Several Authors studied the effectiveness of mutation analysis performed on biopsies or fine needle aspiration (FNA) samples. These studies, along with technological improvements in laboratory methods, confirmed the effectiveness of EGFR mutation analysis in small tumoral samples. This finding was confirmed also in our study. Malapelle et al. in a recent publication compare EGFR mutation analysis in 318 histology samples with that performed on 364 cytology specimens; the Authors registered the 8.5% and 8.8% of mutations in the former and later cases respectively[60].

Unexpectedly, we found significantly higher percentages of neoplastic cells in biopsy specimens, rather than in surgical specimens. Similarly, in the experience of Kim et al. cytological specimens were found to be superior to biopsy specimens in detecting EGFR mutations[61]. These findings suggest that it is not the quantity of neoplastic tissue available that determines the quality of mutational analysis, but the quality of the sample itself in terms of neoplastic and health cells composition. Indeed, one other finding in our study was the statistically significant higher incidence of EGFR mutations in samples composed for more than 50% by neoplastic cells.

The impact of the quantity of neoplastic cells on mutation analysis has been never studied thoroughly in the past, and only empirical and sporadic data are available. Recommendations of several scientific societies on the minimum of neoplastic cells required in the specimen for an adequate mutation analysis are generally based on such data. The Italian guidelines produced recently by a

collaboration of three different scientific societies (AIOM – SIAPEC – IAP) recommend: if standard mutational analysis procedures are used (direct sequencing) it is suggested the sample to be composed of at least 50% of neoplastic cells[62]. As we mentioned before, in our experience more than 85% of the samples examined comprised more than 50% of neoplastic cells.

The proportions of exon-18, exon-19 and exon-21 mutations found in our experience, were similar to those reported in literature for other Caucasian populations[39]. These mutations were more frequent, other than in women and non-smokers, in middle aged patients. In particular, the 21% of the patients in the fourth decade of life presented EGFR mutations, while the corresponding figures were around the 10% in the following decades. This finding should be better investigated in future studies to comprehend its pathophysiological background and clinical utility.

CONCLUSIONS

Our data suggest that the incidence of EGFR mutations among North Sardinian patients with lung adenocarcinoma is similar to those reported in literature for other Caucasian populations in developed countries; conversely, reported incidences in Asiatic populations are higher. The incidence rates of these mutations were higher in females and never smokers than in males and active or former smokers, further confirming literature data. Current practice in EGFR mutation analysis in North Sardinia consists with existing clinical practice guidelines, thanks to a multidisciplinary approach to lung cancer sufferers, obtained through the collaboration of the local clinical, surgical, pathological and biomolecular services.

From a technical point of view, our data evidenced that molecular analysis for EGFR mutations in lung adenocarcinomas can be optimally performed in either primitive and metastatic tissues, regardless of the method of sample retrieval (biopsy or surgery). The percentage of neoplastic cells in the specimen examined seems to be relevant for the quality of the analysis: samples with more

than 50% of neoplastic cells are more reliable for mutation analysis, but further investigations are necessary to confirm this finding, as in great part of the articles published on the topic the percentages of the malignant cells in the samples are not reported. Furthermore, a high percentage of EGFR mutations were found in our area in patients in the fourth decade of life; this finding must be better investigated in the future to comprehend its pathophysiological background and clinical utility.

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