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Research Article

Expression Levels of Some Antioxidant and Epidermal Growth Factor Receptor Genes in Patients with Early-Stage Non-Small Cell Lung Cancer

Giuseppe De Palma,[1] Paola Mozzoni,[2,3] Olga Acampa,[2,3] Eveline Internullo,[4] Paolo Carbognani,[4] Michele Rusca,[4] Matteo Goldoni,[2,3] Massimo Corradi,[3] Marcello Tiseo,[5] Pietro Apostoli,[1] and Antonio Mutti[3]

[1] *Department of Experimental and Applied Medicine, Section of Occupational Health and Industrial Hygiene, University of Brescia, Piazzale Spedali Civili, 1, 25123 Brescia, Italy*
[2] *ISPESL Research Center at the University of Parma, via Gramsci 14, 43100 Parma, Italy*
[3] *Laboratory of Industrial Toxicology, Department of Clinical Medicine Nephrology and Health Sciences, University of Parma, via Gramsci 14, 43100 Parma, Italy*
[4] *Thoracic Surgery Unit, University of Parma, via Gramsci 14, 43100 Parma, Italy*
[5] *Division of Medical Oncology, University of Parma, via Gramsci 14, 43100 Parma, Italy*

Correspondence should be addressed to Giuseppe De Palma, depalma@med.unibs.it

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This study was aimed at: (i) investigating the expression profiles of some antioxidant and epidermal growth factor receptor genes in cancerous and unaffected tissues of patients undergoing lung resection for non-small cell lung cancer (NSCLC) (cross-sectional phase), (ii) evaluating if gene expression levels at the time of surgery may be associated to patients' survival (prospective phase). Antioxidant genes included heme oxygenase 1 (*HO-1*), superoxide dismutase-1 (*SOD-1*), and -2 (*SOD-2*), whereas epidermal growth factor receptor genes consisted of epidermal growth factor receptor (*EGFR*) and v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2 (*HER-2*). Twenty-eight couples of lung biopsies were obtained and gene transcripts were quantified by Real Time RT-PCR. The average follow-up of patients lasted about 60 months. In the cancerous tissues, antioxidant genes were significantly hypo-expressed than in unaffected tissues. The *HER-2* transcript levels prevailed in adenocarcinomas, whereas *EGFR* in squamocellular carcinomas. Patients overexpressing *HER-2* in the cancerous tissues showed significantly lower 5-year survival than the others.

1. Introduction

Lung cancer, in particular non-small cell lung cancer (NSCLC), is the leading cause of cancer related deaths worldwide, given its incidence and poor prognosis mainly due to delay in diagnosis [1]. Molecular heterogeneity in the genomics and/or proteomics of NSCLC may underlie a different clinical outcome and response to therapy of patients with similar clinical stage and histopathology. Among molecular markers, expression profiles of antioxidant genes and proto-oncogenes, identified by microarrays or quantitative reverse transcriptase PCR (qRT-PCR), may significantly affect the clinical outcome of NSCLC [2–4].

Oxidative stress plays a key role in both development and progression of NSCLC. Reactive oxygen species (ROS) can induce DNA damage that, if unrepaired, may lead to disruption of gene transcription as well as to interference with DNA methylation [5, 6]. On the other hand, ROS may up-regulate transcription factors as MAP-kinase/AP-1 and NF-kB that, in turn, induce the expression of several genes involved in key cellular pathways [7, 8]. Among these, there is heme oxygenase-1 (HO-1), a microsomal heat shock protein that catalyzes the first and rate-limiting step in heme catabolism and shows high inducibility by several stimuli [9]. Expressed in airway epithelial cells and alveolar macrophages, it displays both a cytoprotective

and antioxidant role [10]. Cytosolic and mitochondrial superoxide dismutases (SOD-1 and SOD-2, resp.) play also a prominent role in the pulmonary antioxidant defense system [11], catalyzing the dismutation of superoxide anion to hydrogen peroxide and water. Experimental evidence is available showing that ROS may induce the ligand independent phosphorylation of the Epidermal Growth Factor Receptor (EGFR) through oxidized src kinases [12, 13]. Moreover, the expression of the receptor is induced in conditions of oxidative stress [14]. The EGFR belongs to the ErbB family of transmembrane receptors including at least four isoforms, namely erbB-1 (EGFR), erbB-2 (HER-2), erbB-3 (HER3), and erbB-4 (HER4), whose role in lung carcinogenesis as oncogenes is generally accepted [15]. Following the binding of epidermal growth factor (EGF)-like ligands, these receptors homo/hetero-dimerize on the cell surface and activate the cytosolic tyrosine-kinase domain, with downstream stimulation of intracellular signalling pathways, which are essential for cancer development and progression [16, 17]. Both EGFR and HER-2, having an 85% homology in amino acid residues, trigger similar down stream signal event. There are no known ligands for HER-2 that is the preferred heterodimerization partner of this family of receptors [16].

The main aim of the present study was to evaluate differences in the expression profiles of antioxidant (*HO-1, SOD-1, SOD-2*) and epidermal growth factor genes (*EGFR, HER-2*) in both cancerous and unaffected lung tissues of clinical early-stage NSCLC patients.

As a further aim, we evaluated the influence of gene expression levels at the time of surgery on further patients' survival.

2. Materials and Methods

2.1. Study Design. This was an observational study on consecutive patients with a single lung lesion either proven or suspected to be an early clinical stage (I or II) of NSCLC and undergoing lung resection with curative intent. The study was both cross-sectional and prospective in nature. In the cross-sectional phase, we evaluated the gene expression levels in both cancerous and unaffected lung tissues, whereas in the prospective phase, we evaluated the influence, if any, of gene expression levels at the time of surgery on the patients' survival times.

Patients' enrollment proceeded from June to August 2002 and from April 2003 to April 2004, with the interruption being due to a temporary lack of adequate systems of biopsy storage. Exclusion criteria were clinical T3 tumors and a history of malignancy in the previous 5 years. Informed consent was obtained from each patient at the time of enrollment, and sample collection and storage were performed according to the principles laid down in the Declaration of Helsinki (1964). The study received approval by the local Ethics Committee.

Preoperative work-up included medical history and examination, oncological staging and functional evaluation. All patients were staged by chest and abdomen CT scans; in addition, brain CT scan, abdominal ultrasound, or bone scintigraphy were performed if indicated. Any death occurring within 30 days from surgery or during the same hospitalization was defined as postoperative mortality and cases were excluded from survival analysis.

Survival was calculated from the day of surgery to death or last follow-up (1 April 2008), provided that patients did not receive any palliative therapy.

2.2. Sample Collection and Storage. Small pieces (about 0.5–1 cm^3) of lung biopsies, from both cancerous and unaffected tissues of the resected specimen, were collected into tubes containing the RNAlater reagent (Ambion Inc., Austin, TX, USA) and stored at -80°C until analysis. Unaffected tissue samples underwent intraoperative frozen section examination to exclude tumor infiltration.

2.3. RNA Isolation and cDNA Synthesis. Total RNA was isolated by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and digested with DNase I (DNA-*free*, Ambion Inc., Austin, TX, USA). RNA quality was checked by 1% TBE agarose gel electrophoresis, using a denaturing loading buffer (RNA Ladder, New England Biolabs Inc., Beverly, MA, USA). Total RNA quantification was carried out by the RiboGreen probe (Molecular Probes, Eugene, OR, USA) on a Cary Eclipse fluorescence spectrophotometer equipped with a microplate reader (Varian Inc, Scientific Instruments, Palo Alto, CA, USA). cDNA was synthesized using 250–500 ng of total RNA, 250 ng of random exhamer primers (Invitrogen, Carlsbad, CA, USA), and 200 U of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommended experimental conditions.

2.4. Gene Expression. HO-1, SOD-1, SOD-2, EGFR, and *HER-2* gene expression was assessed by Real-Time qRT-PCR on an iCycler iQ Multicolor RealTime PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR mixture contained 2 μL of template cDNA, 400 nM of primers, 100 nM of FAM-conjugated Universal Probe (Roche, Diagnostic Mannheim, Germany), and 2× QuantiTect Probe RT-PCR Master Mix (Qiagen, Hilden, Germany) and water to 25 μL. Table 1 presents the specific primers, spanning the exon-exon junctions, and probes, designed by the ProbeFinder software (Roche Diagnostics, Mannheim, Germany). The amplification protocol consisted of 15 minutes at 95°C followed by 40 cycles at 94°C for 20 s and at 60°C for 1 minute. Duplicate assays were run for each sample and each plate included a standard curve and a negative control.

The relative transcript quantification was calculated by the *geNorm*_algorithm for Microsoft Excel [18] after normalization with β_2*-microglobulin (β_2M), phosphoglycerate kinase 1 (PGK1), succinate dehydrogenase complex subunit A (SDHA), hypoxanthine-guanine phosphoribosyltransferase (HPRT),* and *ribosomal protein L13 (RPL13)* genes, and expressed in terms of arbitrary units (a.u.).

2.5. Statistical Analysis. Statistical analysis was performed by the SPSS 15.0 for Windows software. Variables were not normally distributed, also after logarithmic transformation,

Table 1: Nucleotide sequences for primers and probes used in the present study.

Genes	Forward primers	Reverse primers	Probes
SOD-1	5′TCATCAATTTCGAGCAGAAGG3′	5 CAGGCCTTCAGTCAGTCCTTT3′	5′FAM TGGGGAAG3′DQ
SOD-2	5′TGGTGGAGAACCCAAAGG3′	5′GTCAAAGGAACCAAAGTCACG3′	5′FAM GCTGGAAG3′DQ
HO-1	5′GGGTGATAGAAGAGGCCAAGA3′	5′AGCTCCTGCAACTCCTCAAA3′	5′FAM CATCCAGC3′DQ
EGFR	5′ACACAGAATCTATACCCACCAGAGT3′	5′ATCAACTCCCAAACGGTCAC3′	5′FAM TCTGGAGC3′DQ
HER-2	5′GATCCGGAAGTACACGATGC3′	5′GCTCCGCTAGGTGTCAGC3′	5′FAM TGGTGGAG3′DQ
PGK1	5′GGAGAACCTCCGCTTTCAT3′	5′CTGGCTCGGCTTTAACCTT3′	5′FAM GGAGGAAG3′DQ
B2M	5′TTCTGGCCTGGAGGCTATC3′	5′TCAGGAAATTTGACTTTCCATTC3′	5′FAM CATCCAGC3′DQ
SDHA	5′AGAAGCCCTTTGAGGAGCA3′	5′CGATTACGGGTCTATATTCCAGA3′	5′FAM GGAGGAAG3′DQ
HPRT	5′TGACCTTGATTTATTTTGCATACC3′	5′CGAGCAAGACGTTCAGTCCT3′	5′FAM GCTGAGGA3′DQ
RPL13	5′ACAGCTGCTCAGCTTCACCT3′	5′TGGCAGCATGCCATAAATAG3′	5′FAM CAGTGGCA3′DQ

Legend: FAM = fluorescein; DQ = dark quencher dye.

hence data were analyzed by nonparametric tests. Comparisons between two independent or two related samples were performed by the Mann-Whitney U or the Wilcoxon's test. The ratios of gene expression levels in the cancerous versus the unaffected tissues were recoded into dichotomic variables (values up to or higher than one) that were used to evaluate survival curves by the Kaplan Meyer analysis with log-rank (Mantel-Cox) test. The Cox regression analysis was used to study the effect of multiple covariates on survival curves. Two patients deceased for causes surely unrelated to NSCLC (a postoperative death and pulmonary embolism after discharge) were excluded from analyses of survival.

Table 2: Distribution of gene transcript levels (medians and interquartile (25th–75th) ranges of values in arbitrary units, a.u) in cancerous and unaffected tissues. Results of paired sample analysis (Wilcoxon's test) are shown (P).

Gene transcripts	Cancerous tissue, a.u.	Unaffected tissue, a.u.	P
HO-1	0.10 (0.07–0.15)	0.16 (0.13–0.22)	.031
SOD-1	0.13 (0.09–0.15)	0.18 (0.14–0.19)	.006
SOD-2	0.17 (0.09–0.23)	0.26 (0.18–0.36)	.001
EGFR	0.07 (0.04–0.09)	0.05 (0.03–0.07)	.258
HER-2	0.23 (0.09–0.37)	0.23 (0.16–0.35)	.228

3. Results and Discussion

During the study period, 89 patients were admitted at a single center as affected by a lung lesion proven or suspected to be an NSCLC at clinical stage I or II. Of these: 10 were excluded for clinical stage $T_3N_0M_0$ and 15 for a history of malignancy in the previous 5 years. Nine patients did not give consent to the study, thus 55 were enrolled. In eight cases, the lung lesion was too small (1.5 cm maximum diameter) or completely endobronchial, so that the surgeon did not perform any biopsy in order not to spoil the final pathology exam. Of the 47 patients undergoing biopsy, 19 were weeded out because frozen section on the nodule revealed benign lesions ($n = 7$) or metastases from another primary site ($n = 7$) or carcinoid tumors ($n = 5$). Therefore, 28 couples of biopsies (cancerous and unaffected tissues) constitute the final study sample. There were 5 females and 23 males, mean aged 70 years (range 55–82). Twenty-six out of 28 were either previous or current smokers, with a mean tobacco exposure of 50.5 pack/years. Twenty-four lobectomies, three pneumonectomies, and one anatomical segmentectomy were performed, associated with standard lymphadenectomy. There were 14 adenocarcinomas (ADC), 13 squamous cell carcinomas (SCC), and 1 undifferentiated carcinoma. Despite all patients were clinically stage I or II at preoperative examinations, final pathology [19] revealed the following: stage I in 20 cases (6 stage Ia and 14 stage Ib), stage II in 5 (1 IIa and 4 IIb), and stage IIIa in 3. None of the patients received adjuvant therapy or any patient was lost at follow-up. At the time of data analysis (70 months after the first patient was enrolled), 14 (50%) of the patients had died: 12 by disease recurrence and two by tumor unrelated causes, by postoperative death and pulmonary embolism after discharge, respectively. The overall 5-year survival was 50% and it became 56% if considering only stage I and II patients. Median follow-up for the 14 patients alive was 58.9 months (range 48–70).

Table 2 shows the average levels of investigated transcripts both in cancerous and unaffected lung tissues. In two cases, we failed to characterize the transcript levels, because of poor quality RNA.

The *HO-1, SOD-1,* and *SOD-2* transcript levels were significantly lower in the cancerous tissue, whereas no significant difference was apparent for both *EGFR* and *HER-2* mRNA levels. After stratification of patients by histopathology (25 subjects, excluding the only patient with diagnosis on undifferentiated carcinoma), some difference was apparent only for *EGFR* and *HER-2* transcripts. The levels of *HER-2* mRNA were significantly higher in ADC than SCC tumor samples (median values of 0.38 (interquartile range 0.24–0.51) a.u. versus 0.10 (0.07–0.13) a.u., $P < .001$), whereas the difference was not significant for the *EGFR* transcript (Figure 1(a)). Figure 1(b) shows the ratios of *EGFR* and *HER-2* transcript levels in the cancerous versus unaffected tissues. *EGFR* levels prevailed significantly in subjects with

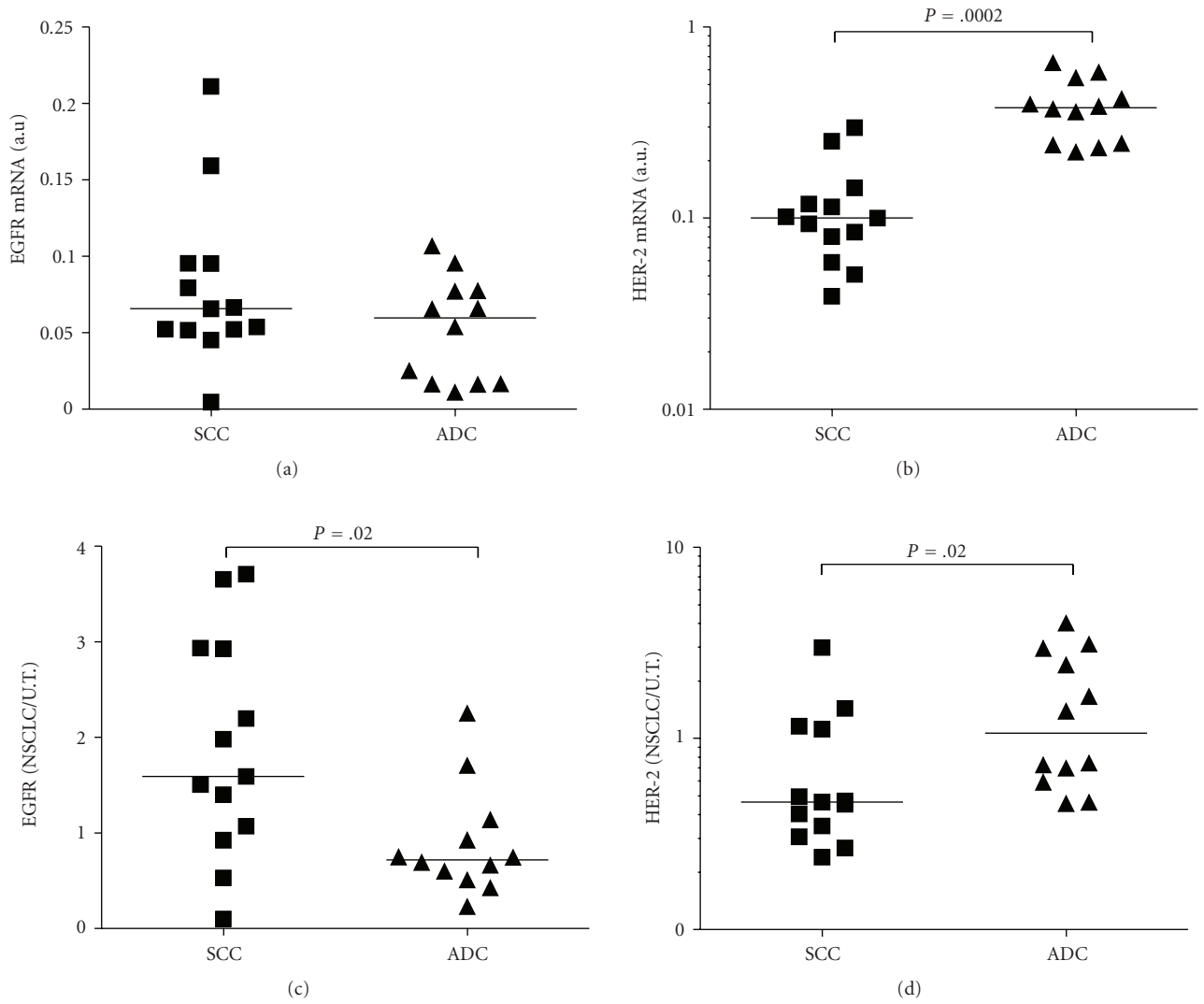

Figure 1: (a) *EGFR* and *HER-2* transcript levels (as arbitrary units, a.u.) in squamocellular carcinoma (SCC) and adenocarcinoma (ADC) samples. Lines represent medians of values. (b) Expression ratio levels of the same genes in the cancerous (C.T.) versus unaffected tissues (U.T.). Lines represent medians of values.

SCC as compared to those with ADC (median values of 1.59 (interquartile range 1.00–2.93) a.u. versus 0.72 (0.53–1.08) a.u., $P = .019$), whereas the opposite held true for *HER-2* (median values of 1.06 (interquartile range 0.62–2.82) a.u. versus 0.46 (0.33–1.14) a.u., $P = .019$).

Then, we proceeded to analyze the survival times on 24 patients (in two cases, gene expression results were unavailable and two cases were excluded, given their death by cancer unrelated causes). Table 3 shows that subjects over-expressing *HER-2* in the cancerous tissue as compared to the unaffected one displayed survival times about three times lower, on average, than the rest of subjects. In agreement with this, 5-year survivors showed significantly lower *HER-2* transcript levels in the cancerous versus the unaffected tissue than patients dead within 5 years from surgery (medians (interquartile ranges) of 0.48 (0.32–0.73) a.u. versus 1.39 (0.70–2.95) a.u., $P < .05$).

The effect of the *HER-2* transcript ratio on survival was confirmed by the Kaplan Meier analysis (Figure 2). A Cox regression model including also age, sex, tumor stage, and histology as covariates confirmed the role of *HER-2* transcript ratio as main factor ($P = .012$) affecting the patients' survival, whereas other covariates showed no effect. In the same model, the strength of association between longer survival times and *HER-2* transcript ratio values higher than 1 was 11.42 (95% confidence interval: 1.74–74.84).

The present study shows that the expression levels of genes displaying antioxidant and cytoprotective properties, such as *SOD-1, SOD-2,* and *HO-1,* are depressed in lung cancer as compared to the unaffected lung tissue. These results indirectly confirm the association of oxidative stress with the neoplastic phenotype. Superoxide dismutases are generally regarded as first line antioxidant enzymes, given their unique role in detoxification of superoxide radicals. Previous studies investigating on SOD expression in human lung cancer produced variable results (reviewed in [20]). With the exception of one study, finding raised *SOD-2* (and not *SOD-1*) transcript levels (by Northern blot analysis) in

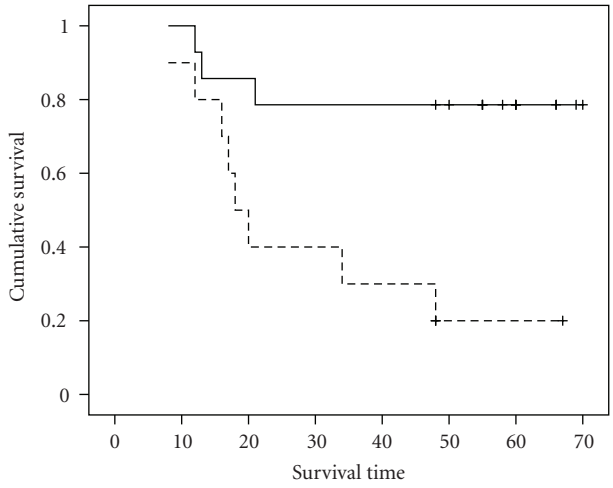

Figure 2: Kaplan-Meier plot of survival times in NSCLC patients classified by expression ratio levels of *HER-2* transcript in cancerous versus unaffected tissues (up to or higher than one). The dashed line represents subjects over-expressing *HER-2* mRNA in the cancerous versus unaffected tissues, whereas the continuous line indicates subjects with lower expression levels of *HER-2* mRNA in cancerous versus unaffected tissue samples. Crosses indicate censored subjects, represented by patients still alive at 1st April 2008.

Table 3: Average survival times in subjects classified according to the expression levels of different transcript in cancerous (C.T.) versus unaffected (U.T.) tissues. Medians and interquartile (25th–75th) ranges of values are shown, as well as the results of the Mann-Whitney U test (*P*).

Transcripts	Survival (months)		*P*
	C. T. / U.T >1	C. T. / U.T ≤ 1	
HO-1	48.00 (33.00–63.00)	48.00 (16.50–59.00)	.659
SOD-1	52.50 (11.00–60.00)	48.00 (17.50–63.00)	.865
SOD-2	18.00 (14.50–57.00)	49.00 (20.25–59.50)	.303
EGFR	51.50 (17.75–59.50)	48.00 (16.50–64.50)	.843
HER-2	**19.00 (15.00–48.00)**	**56.50 (41.25–66.00)**	**.019**

the cancerous, as compared to unaffected tissue [21], the others investigated either whole SOD activity, irrespective of isoenzymes, or SOD proteins (by immunohistochemistry (IHC) or Western blot). Some criticism has been raised about the specificity of antibodies to SOD-2 [20]. Our results are consistent with the experimental observation that SOD activity may suppress the malignant phenotype [20].

The role of HO-1 in carcinogenesis has not yet been well elucidated [22]. Our data support the hypothesis that the expression of the enzyme is defective in lung cancer tissue and are in agreement with a recent study [23] showing reduced HO-1 expression (by IHC methods) in the alveolar macrophages of patients with NSCLC, as compared to controls.

Overexpression of EGFR and HER-2 receptors in NSCLC samples has been reported in many series, ranging from 43 to 89% and from 16 to 57% of tumors and patients, respectively (reviewed in [24]). Inconsistent results may reflect differences in analytical techniques, procedures, and study populations. Several methods are available to evaluate the tissue expression of these receptors, including those looking at proteins (IHC, Western blotting or enzyme immunoassay), RNA (Northern blot analyses and RT-PCR), or DNA (fluorescence in situ hybridization (FISH) or quantitative PCR) levels. The most commonly used technique was IHC, whose standardization is far to be achieved [25]. The Real-Time qRT-PCR we used for our analyses is an accurate technique that may allow the direct comparison of expression levels of target genes in both the cancerous and unaffected tissues. Our study, even demonstrating similar expression profiles of *EGFR* and *HER-2* in cancerous and unaffected tissues, confirmed that *EGFR* is overexpressed in SCC, as compared to ADC samples, whereas *HER-2* displays an opposite behaviour [25, 26]. This may have therapeutic consequences, as both the proteins have been validated as clinically relevant targets, and several different types of agents inhibiting these receptors are currently under development [27].

In this study, the overall five-year survival rate (56% considering only patients at stages I and II) was consistent with previous observation [19, 28]. However, the *HER-2* expression ratio in the cancerous versus the unaffected tissue was a significant predictor of the 5-year prognosis. At the time of surgery, five-year survivors showed values of *HER-2* transcript ratio that were on average about 30% of the values observed in patients undergoing death. At the end of the follow-up period, subjects over-expressing the receptor in the cancerous tissue were deceased by 80%, whereas those with lower expression ratios were still alive by 80%. The Cox multivariate analysis allowed us to exclude any interference by tumor stage, histology, and age. A main limitation of this study was its small sample size. However, the extent of deviation from expected 5-year survival in both the patient groups ensures the statistical power of the results (alpha = 5%, beta = 80%). Our data are in agreement with previous studies, mostly relying on IHC characterizations and giving a meta-analytical hazard ratio of 1.5 (95% confidence interval 1.29–1.86) for worse prognosis associated to HER-2 overexpression [24]. The mechanisms linking *HER-2* expression to a bad prognosis rely on a pleiotropic cascade of effects secondary to HER-2 activation. These include (i) increase of cell proliferation and survival and perturbation of differentiation, dependent on the activation of the phosphatidylinositol 3-kinase-AKT and the ras/mitogen-activated protein kinase pathways [29, 30], (ii) increased angiogenesis, as a consequence of the ability of HER-2 to modulate the equilibrium between proangiogenic (VEGF, IL-8, angiopoietin-2) and antiangiogenic factors [31].

4. Conclusions

Obtained results demonstrate that in biopsy samples from clinical early NSCLC, the expression levels of some antioxidant genes are depressed as compared to the surrounding healthy tissue. Interestingly, the patients' prognosis shows a significant inverse association to the ratio of *HER-2* transcript levels in the cancerous versus the unaffected tissue.

However, owing to the small sample size, caution must be exercised in the interpretation of results that are to be

considered as preliminary. Further confirmation by larger studies is needed, before observed findings may be translated into biomarkers useful to improve clinical managing of NSCLC cases.

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