RESEARCH ARTICLE

Oxidative stress associates with aggressiveness in lung large-cell carcinoma

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Abstract Oxidative stress is involved in many cancer-related processes; however, current therapeutics are unable to benefit from this approach. The lungs have a very exquisite redox environment that may contribute to the frequent and deadly nature of lung cancer. Very few studies specifically address lung large-cell carcinoma (LCC), even though this is one of the major subtypes. Using bioinformatic (in silico) tools, we demonstrated that a more aggressive lung LCC cell line (HOP-92) has an overall increase activity of the human antioxidant gene (HAG) network ($P=0.0046$) when compared to the less aggressive cell line H-460. Gene set enrichment analysis (GSEA) showed that the expression of metallothioneins (MT), glutathione peroxidase 1 (GPx-1), and catalase (CAT) are responsible for this difference in gene signature. This was validated in vitro, where HOP-92 showed a pro-oxidative imbalance, presenting higher antioxidant enzymes (superoxide dismutase (SOD), CAT, and GPx) activities, lower reduced sulfhydryl groups and antioxidant potential, and higher lipoperoxidation and reactive species production. Also, HAG network is upregulated in lung LCC patients with worst outcome. Finally, the prognostic value of genes enriched in the most aggressive cell line was assessed in this cohort. Isoforms of metallothioneins are associated with bad prognosis, while the thioredoxin-interacting protein (TXNIP) is associated with good prognosis. Thus, redox metabolism can be an important aspect in lung LCC aggressiveness and a possible therapeutic target.

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Introduction

Oxidative stress is involved in many processes during carcinogenesis; however, it is not yet possible to benefit patients with this approach [1, 2]. Free radicals are believed to initiate tumorigenesis, causing DNA mutations, and promote cancer by regulating cell survival, proliferation, apoptosis [3, 4], angiogenesis [5], migration, and metastasis [6, 7]. Although it was thought that antioxidant treatment could impair cancer cell homeostasis [8], several studies showed that this approach can enhance tumor progression [2, 9]. Since cancer cells survives in an already highly stressed environment, pro-oxidative treatments could push cancer cells over the edge via selective toxicity [1]. Thus, the way cancer responds to oxidative stress is still in debate.

The lungs have a unique redox balance, since it is exposed to a variety of both endogenous and exogenous oxidants that can contribute to tumor promotion [10]. These factors include environmental pollutants, tobacco smoke, high oxygen pressure [11], and reactive species (RS) from pro-inflammatory cells in the pulmonary circulation [12]. Moreover, lung cancer patients were found to have elevated oxidative stress markers in peripheral blood [13], erythrocytes [14, 15], epithelium lining fluid [16], breath condensate [17], and tumor biopsies [18–20], and the inadequate ingest of antioxidants may constitute a risk factor for lung cancer [21]. Despite this, the association between a redox imbalance and lung LCC was never investigated.

Lung cancer is the most prevalent and deadly malignancy worldwide [22] and often classified into small-cell and non-small-cell lung cancer (NSCLC). Of the latter, large-cell

carcinoma (LCC) is one of its most common subtypes (5–10 % of all lung cancer cases). Despite its importance, there are few studies focusing on lung LCC and its classification by the WHO is vague: undifferentiated NSCLC that lacks the cytological and architectural features of small-cell lung cancer and glandular or squamous differentiation [23]. Besides, half diagnosed lung LCC have been shown to belong to another category when molecular markers were used [24, 25].Therefore, studies focusing specifically on lung LCC are needed [26].

In light of the above, this study aimed to establish a relationship between oxidative stress and lung LCC aggressiveness. To achieve this, we characterized the aggressiveness of two human lung LCC cell lines and found that HOP-92 is more aggressive than H-460. Then, we investigated the redox profile of the cell lines with bioinformatic and in vitro tools. These demonstrated that the most aggressive cell line has a pro-oxidative imbalanced profile and that changes in the redox environment can modulate the behavior of the cells. Also, human antioxidant gene (HAG) network is upregulated in lung LCC patients with worst outcome and genes enriched in the most aggressive cell line have prognostic value in this cohort. Thus, redox metabolism can be an important aspect in lung LCC aggressiveness and a possible therapeutic target.

Materials and methods

Cell lines and chemicals

The human lung LCC cell lines H-460 and HOP-92 were obtained from the NCI-Frederick cell line repository. Exponentially growing cells were cultivated in RPMI 1640 medium (Invitrogen®) containing 10 % fetal bovine serum (FBS), amphotericin B (1 μg/mL), and garamycin (50 μg/L) at 37 °C in a humidified atmosphere of 5 % of CO_2.

Cellular aggressiveness

The invasion index was measured with the BioCoat Matrigel Invasion Chamber System (BD Bioscience®). Briefly, cells were seeded in the upper wells, while the chemoattractant (RPMI medium with 10 % of FBS) was added to the lower wells. After 22-h incubation, the movement of cells through the pore was determined. Cells that penetrated to the underside surfaces of the inserts were fixed and stained with HeMa3 staining kit (Fisher Scientific®) and counted under the microscope. Cells were considered migratory when moved through uncoated pores and invasive when moved through Matrigel-coated pores. Data are expressed as the percentage of invasive/migratory and expressed as "invasion index."

Multidrug resistance was determined based on drug dose-response curves of cisplatin, carboplatin, daunorubicin, doxorubicin, 5-fluorouracil, hydroxyurea, and taxol (Sigma® Chemical Co.) using the sulforhodamine B (SRB) assay, following NCI-60 protocol [27].

### HAG network and microarray datasets

The HAG network was designed to cluster functional gene network to facilitate high-throughput analysis of redox processes [28]. HAG is composed of 63 genes whose products are thiol-containing proteins or enzymes that react directly with reactive species and is subclassified into three functional groups: peroxidases, superoxide dismutases, and thiol-containing redox proteins (Fig. 1a).

Microarray expression profile was extracted from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). For the comparison with the human lung LCC cell lines H-460 and HOP-92, GSE5846 dataset was used. For the validation including H661, GSE4824 and GSE14925 datasets were used. For cohort analysis, we used a microarray dataset with 24 lung LCC specimens containing survival details (GSE37745).

Differential gene expression and enrichment analysis

Differential gene expression was evaluated using ViaComplex® software [29]. To determine the significantly altered groups of functionally associated genes (GFAGs), ViaComplex uses re-sampling analysis with replacement (bootstrapping) in order to estimate the sampling distribution of both relative diversity and relative activity in the microarray dataset. Given that this analysis considers genes in the context of functional groups, the statistical design is constructed to compare groups of genes. The raw P values from the bootstrap analysis are controlled for multiple comparisons by false discovery rate (FDR) analysis. This procedure is used to identify GFAGs exhibiting significant differential expression with a FDR no greater than 5 % (i.e., a 5 % FDR indicates that among all GFAGs identified as being differentially expressed, 5 % of them are truly not significant) [30].

Gene set enrichment analysis (GSEA) was used to identify genes that contribute individually to global changes in expression levels in a given microarray dataset. GSEA considers experiments with genome-wide expression profiles from samples belonging to two classes (i.e., more aggressive and less aggressive cancer cells). Genes are ranked based on the correlation between their expression and the class distinction by using a suitable metric. Given a prior defined set of genes (i.e., HAG network), the goal of GSEA is to determine whether the members of these set of genes are randomly distributed or primarily found at the top or bottom of the ranking [31].
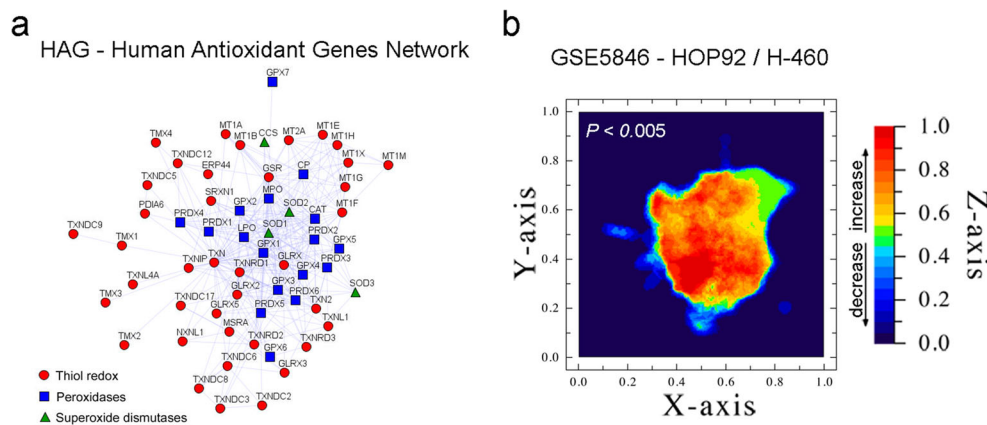
Fig. 1 Expression of human antioxidant gene (HAG) network in human lung large-cell carcinoma (LCC) cell lines. **a** STRING representation of HAG network gene interactions. **b** Landscape analysis demonstrating elevated expression of HAG network in HOP-92 compared to H-460 (GSE5846 dataset), generated with ViaComplex® V1.0. Color gradient (Z-axis) represents the relative functional state mapped onto graph according to the data input from the lung LCC HOP-92-a versus H-460-b, where $z=a/(a+b)$. P value refers to bootstrap analysis comparing cell lines

Redox parameters

Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity was measured by inhibition of superoxide-dependent epinephrine auto-oxidation at 480 nm [32]. Catalase (CAT) (E.C. 1.11.1.6) activity was measured by H_2O_2 consumption at 240 nm. Glutathione peroxidase (GPX) (E.C. 1.11.1.9) activity was measured by NADPH oxidation at 340 nm [33].

Non-enzymatic antioxidant potential was determined by total radical-trapping antioxidant potential (TRAP) assay [34]. Sulfhydryl group (–SH) level was determined with 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon412$ nm$=27,200$/M/cm) and expressed as nanomoles of –SH per milligram of protein. Thiobarbituric acid reactive species (TBARS) assay was used as a lipoperoxidation index. TBARS were assayed at 532 nm and expressed as nanomoles of MDA equivalents per milligram of protein. 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) oxidation was used to determine intracellular generation of RS in a 96-well plate reader (Spectra Max GEMINI XPS, Molecular Devices®).

Proliferation and growth inhibition assays

Cells were treated with active or heat-inactivated CAT, and cell growth inhibition was evaluated for 72 h with SRB assay. To investigate if the growth inhibition by CAT was reversible, the assay was repeated removing CAT. The effect of bolus amount of H_2O_2 addition on cell growth was also evaluated.

Statistical analysis

Cell line data are expressed as means±SEM of at least three independent experiments carried out in triplicate, and Student's t test was used ($P<0.05$) (GraphPad® Software 5.0). Expression analysis were evaluated as mentioned above. Survival graphs were made in GraphPad® Software 5.0, and log-rank (Mantel-Cox) test and hazard ratio (Mantel-Haenszel) were obtained.

Results

Cellular aggressiveness

Comparing in vitro invasion index and multidrug resistance between the two human lung large LCC cell lines, HOP-92 was established as the most aggressive (Table 1). HOP-92 cells had 6-fold higher invasive index and a significantly higher resistance to all seven drugs evaluated (2.79–40.11-fold increase in drug GI$_{50}$ values).

HAG expression

The most aggressive cell line (HOP-92) has higher expression of antioxidant genes, as demonstrated in the landscape map ($P=0.0046$) (Fig. 1b). To confirm this finding, we looked for another lung LCC cell line with freely available expression data and direct aggressiveness comparison between two or more cell lines as part of one published study. Then, we compared HAG expression in H-460 and H661 lung LCC cell lines. Among the cell lines used in this study, H661 is the less aggressive one, since H-460 has higher migratory behavior, higher expression of the pro-angiogenic protein EphA4, and is more resistant to radiotherapy and the antineoplastic agent AZD1152-HQPA [35, 36]. Once more, the more aggressive cell line has a higher expression of HAG (Fig. S1).

The genes that specifically contribute to enrichment in HOP-92 compared to H-460 are summarized in Table 2 and

Table 1 Aggressiveness of human lung large-cell carcinoma cell lines

	Large-cell carcinoma cell lines		
	H-460	HOP-92	Fold increase
Invasiveness (invasive/migratory cells)			
Invasion Index	2.15 ± 0.3	**12.48 ± 2.6****	5.80
Multidrug resistance (GI$_{50}$ value) (μM)			
Cisplatin	0.90 ± 0.11	**2.51 ± 0.5***	2.79
Carboplatin	23.9 ± 4.9	**75.4 ± 18.3***	3.15
Daunorubicin	0.027 ± 0.007	**0.109 ± 0.03***	4.04
Doxorubicin	0.029 ± 0.01	**0.123 ± 0.03***	4.24
5-Fluorouracil	4.41 ± 1.35	**176.9 ± 65.4***	40.11
Hydroxyurea	315.9 ± 11.1	**713.4 ± 98****	2.26
Taxol	0.026 ± 0.021	**0.095 ± 0.02***	3.65

Data are presented as means±SEM of at least four independent experiments ($n=4$). Invasion index is "invading cells/migrating cells." Bold values indicate higher values in each comparison

*$P<0.05$; **$P<0.01$ (Student's t test)

include metallothioneins, peroxidases, and components of the thioredoxin system (obtained with GSEA).

Oxidative stress in lung LCC cell lines

To validate in silico results, several redox parameters were evaluated in vitro in the lung LCC cell lines (Fig. 2). We found a significant upregulation in all antioxidant enzyme (AOE) activities in the most aggressive cell line. HOP-92 has higher activities of SOD, CAT, and GPx (1.83, 4.76, and 2.1-fold increase, respectively) (Fig. 2a). This suggests an adaptation to higher levels of RS. Consistently with this, HOP-92 was found to have higher DCF oxidation rate indicating elevated production of RS (Fig. 2b). On the other hand, basal

lipoperoxidation (TBARS levels) was found to be 2.26-fold higher and the total antioxidant capacity (TRAP) and the levels of reduced sulfhydryl groups (–SH) in HOP-92 cells (Fig. 2c) were found to be decreased. Collectively, this indicates that despite the enzymatic adaptation, the most aggressive phenotype has higher levels of intracellular oxidative stress.

Also, CAT treatment caused a dose-dependent inhibition of cell's growth and CAT washout restored proliferation. Finally, the treatment with sublethal dose of H_2O_2 increased growth of HOP-92, but not H-460 (Fig. S2). Therefore, the redox environment can modulate the behavior of cancer cells.

Oxidative stress in clinical lung LCC patients

Ultimately, the value of the pro-oxidative imbalance was tested in a patient cohort. In accordance with the previous findings, HAG was found upregulated in patients with worst outcome (Fig. 3a).

Also, the prognostic value of the genes enriched in the most aggressive cell lines was assessed. Different isoforms of metallothioneins (MT1F, MT1G, MT1M, and MT1X) were found to be associated with bad prognosis, while TXNIP was associated with good prognosis (Fig. 3b).

Table 2 Genes from human antioxidant gene (HAG) significantly enriched in most aggressive lung large-cell carcinoma cell line (HOP92)

Gene symbol	Gene name
CAT	Catalase
GPX1	Glutathione peroxidase 1
MT1E	Metallothionein 1E
MT1F	Metallothionein 1F
MT1G	Metallothionein 1G
MT1H	Metallothionein 1H
MT1M	Metallothionein 1M
MT1X	Metallothionein 1X
MT2A	Metallothionein 2A
SEPP1	Selenoprotein P, plasma, 1
TXNIP	Thioredoxin interacting protein

Data generated with gene score enrichment analysis (GSEA) comparing HOP92 versus H-460 transcripts obtained from GSE5846 dataset (Gene Expression Omnibus) ($P<0.05$)

Discussion

Despite evidence of oxidative stress involvement in several cancer-related processes, such as resistance to chemotherapy [37, 38], angiogenesis [5], cellular immortalization [7], and cell death [39, 40], it is disappointing that to date no

Fig. 2 Redox characterization of lung large-cell carcinoma cell lines. Activity of antioxidant enzymes SOD, CAT, and GPx (**a**), ROS generation (**b**), and non-enzymatic parameters (**c**). Data are means±SEM of at least three independent experiments ($n=3$), performed in triplicate. *$P<0.05$ or **$P<0.01$ (Student's t test between cell lines)
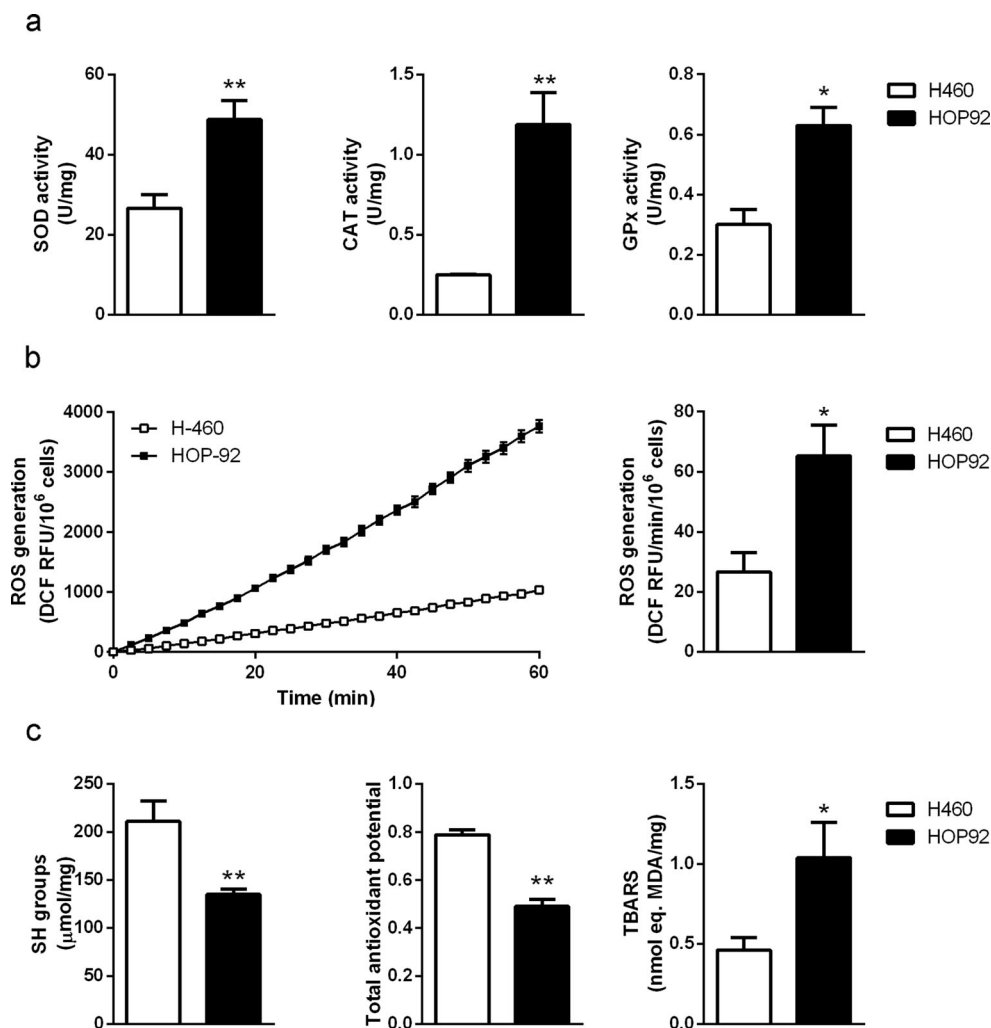
antioxidant approach has been successfully translated to the oncologic clinical setting [8]. Additionally, it has been shown that lung cancer patients have elevated oxidative stress markers in peripheral blood [13], erythrocytes [14, 15], epithelium lining fluid [16], breath condensate [17], and in tumor biopsies [18–20], and the inadequate ingestion of antioxidants constitutes a risk factor for lung cancer development [21]. Here, we demonstrated that the most aggressive cell line has a pro-oxidative imbalance and that oxidative stress can modulate tumoral cell's behavior. Also, this imbalance was confirmed in a lung LCC cohort and genes enriched in the most aggressive cell line were shown to have prognostic value. The results will be further explored below.

Redox biology is intricately overlapped with several metabolic pathways, so we felt this was best evaluated using a systems biology approach [41]. We demonstrated an association between tumoral aggressiveness and higher expression of antioxidant genes using an in vitro cell system and in silico tools. This was corroborated by in vitro redox characterization.

Several isoforms of MT were found to be upregulated in the most aggressive cell line and were associated with bad prognosis. We found it to be upregulated also in a more aggressive lung adenocarcinoma cell line [3]. This is in accordance with data showing that MT are associated with drug resistance, lung cancer progression, and poor patient outcome [42]. Therefore, metallothioneins seems to be a very good candidate for lung cancer studies focusing on oxidative stress.

The thioredoxins (TXN) are antioxidants usually overexpressed and correlated with bad prognosis in several malignancies including lung cancer [43, 44]. On the other hand, the TXN inhibitor thioredoxin-interacting protein (TXNIP) has a negative correlation with TXN, being found underexpressed in tumors and correlating with good prognosis. Moreover, TXNIP is considered a tumor suppressor involved in reduced tumor growth, metastasis, and angiogenesis in different types of both solid tumors (as breast, gastric, thyroid, bladder, and liver) and leukemia [45–50]. However, we found TXNIP to be overexpressed in the most aggressive cell line. Despite the clear contrast with the abovementioned, this is not the first time we report this. TXNIP was found to be overexpressed in more aggressive lung adenocarcinoma cell line and patients [3]. Also, TXNIP overexpression results in

Fig. 3 Pro-oxidative imbalance in lung LCC cohort. Patients with worst outcome have a higher expression of the human antioxidant gene (HAG) network (**a**). Five genes that are enriched in the most aggressive lung LCC cell lines have prognostic value; metallothioneins (MT1F, MT1G, MT1M, and MT1X) are associated with bad prognosis while TXNIP is associated with good prognosis (**b**). Transcript profiles of human lung LCC patients were obtained from Gene Expression Omnibus (GEO). The 24 lung LCC patients were divided according to the value of expression of each gene in two groups: the top or below the median of the entire cohort (GSE37745). Survival graphs were made in GraphPad® Software 5.0, and log-rank (Mantel-Cox) test and hazard ratio (Mantel-Haenszel) were obtained. $P<0.05$. *HR* hazard ratio, *CI* confidence interval
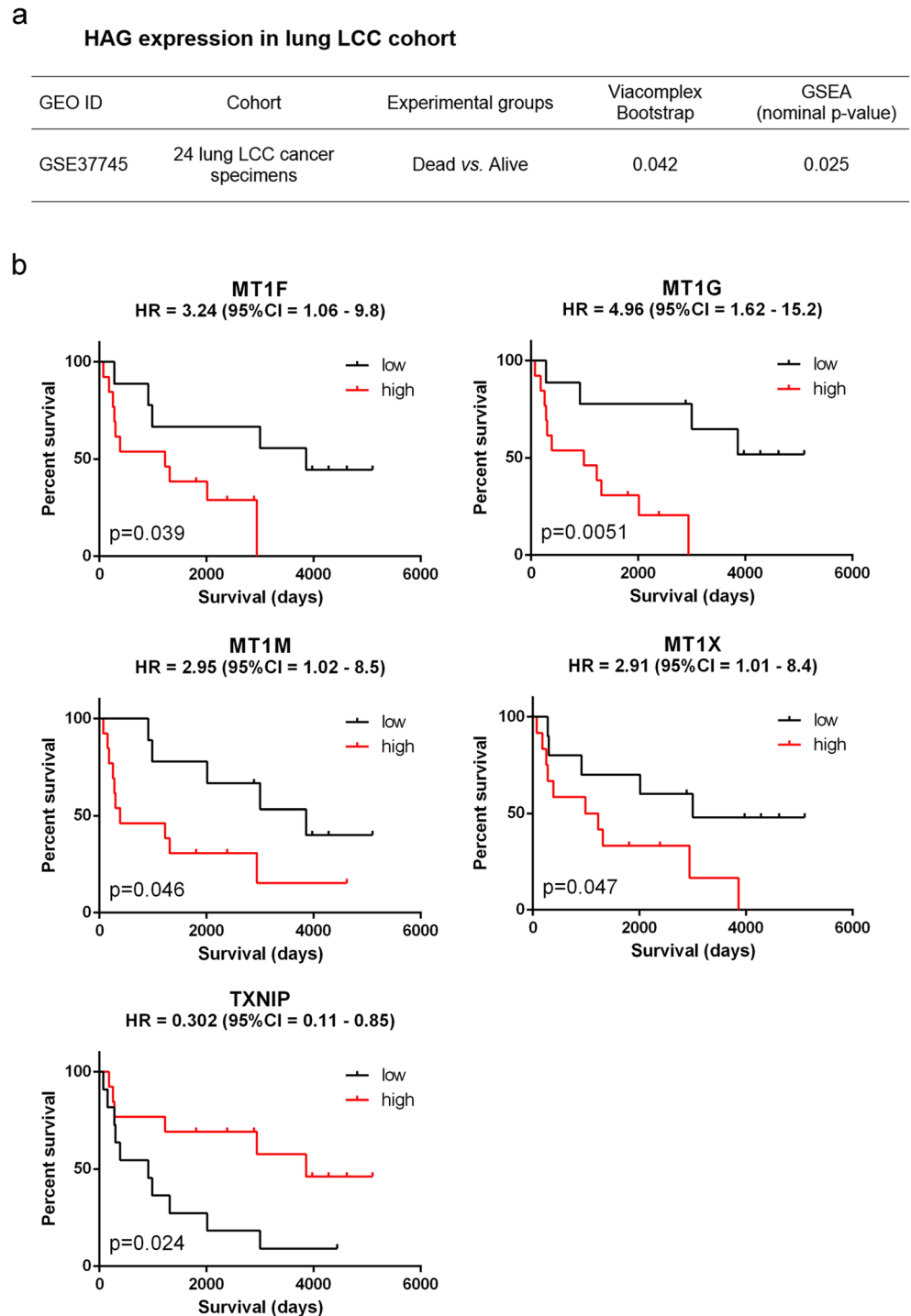
increased levels of reactive species [45], corroborating our main hypothesis. In the meantime, TXNIP associates with good prognosis in the lung LCC cohort tested. Thus, more studies are necessary to fully comprehend how this gene, and the TXN pathway as a whole, can affect lung LCC aggressiveness.

Catalase and GPx were found to have higher expression and activity in the most aggressive cell line and could be an

interesting target for future studies, particularly because both can detoxify H_2O_2. The higher activity and expression of antioxidant enzymes coupled with higher production of ROS suggest that most aggressive lung LCC are adapted to deal with more oxidizing environments. Since malignant tumors are resistant to cell death, they can benefit from ROS stimuli for proliferation and cell growth [51]. In accordance with this, patients with worst outcome presented higher expression of

HAG and the most aggressive cell line was the only one that enhanced its growth rate when treated with H_2O_2. Taken together, this indicates that aggressive lung LCC can have a pro-oxidative imbalance, which could have therapeutic implications.

Nevertheless, recent studies demonstrated that the antioxidants N-acetylcysteine (NAC) and vitamin E increases tumor progression and worsen outcome in in vivo model of lung cancer [2]. On the other hand, catalase overexpression [52] and H_2O_2 scavenging [53] were shown to revert malignant features in different cell lines; mitochondrial-targeted catalase suppresses invasive breast cancer in mice [54] and prevented tumor growth and metastasis in mouse lung studies [55]. Another important detail is the quality of the antioxidant: catalase and other H_2O_2 scavengers have a specific target, while NAC and vitamin E do not. Our data supports the hypothesis that oxidants, especially H_2O_2, fuel the behavior of tumor cells and thus specific antioxidants could impair malignant cell homeostasis by ROS starvation.

To the knowledge of the authors, this is the first study demonstrating an association between redox imbalance and tumor aggressiveness in human lung LCC. Finally, besides consistent evidence of elevated oxidative stress occurring in lung cancer patients, future studies should focus on the specific redox mechanism that mediates tumor aggressiveness for the improvement of lung LCC therapy.

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