Short report

COL1A1, PRPF40A and UCP2 correlate with hypoxia markers in non-small cell lung cancer

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Short title: COL1A1, PRPF40A & UCP2 correlate with hypoxia in NSCLC

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Abbreviations: COL1A1 (collagen 1A1), PRPF40A (RNA–binding and pre-mRNA Processing Factor), UCP2 (Uncoupling Protein 2), HIF1α (Hypoxia-Inducible Factor 1α), VEGFa (Vascular Endothelial Growth Factor), CYGB (Cytoglobin), NSCLC (Non-Small Cell Lung Cancer), SqCLC (Squamous Cell Lung Cancer)

Statement: COL1A1, PRPF40A and UCP2, (downstream effector genes of the novel tumour suppressor gene, cytoglobin) are upregulated in lung cancer and strongly associated with

expression of hypoxia markers. These findings indicate their potential implications in the molecular pathogenesis and clinical outcome of non-small cell lung carcinomas.

Abstract

Collagen 1A1 (COL1A1), RNA–binding and pre-mRNA Processing Factor (PRPF40A) and Uncoupling Protein 2 (UCP2) were recently identified as downstream effector genes of cytoglobin (CYGB), which has been shown to be implicated in tumour biology. Although these three genes have been previously associated with cancer, little is known about their status in lung malignancies. In the present study, we investigated the expression and promoter methylation of COL1A1, PRPF40A and UCP2 in 211 non-small cell lung cancer (NSCLC) and adjacent normal tissue samples. We demonstrate that COL1A1 and PRPF40A mRNAs are significantly overexpressed in NSCLC ($p<1x10^{-4}$), while UCP2 exhibits a trend of upregulation (p=0.066). Only COL1A1 promoter revealed hypermethylation in NSCLCs (36%), which was particularly evident in squamous cell carcinomas (p=0.024) and in the tumours with moderate to good differentiation (p=0.01). We did not observe correlation between DNA methylation and expression of COL1A1. However, mRNA level of COL1A1, as well as PRPF40A and UCP2 exhibited striking association with the expression of hypoxia markers: HIF1α and VEGFa ($p\leq0.001$). In addition, we demonstrate in lung cancer cell lines exposed to hypoxia or oxidative stress that COL1A1 transcription significantly responds to oxygen depletion, while other genes showed modest upregulation in stress conditions. In conclusion, our data revealed that COL1A1, UCP2 and PRPF40A are novel players implicated in the complex network of hypoxia response in the NSCLC.

Introduction

Lung cancer is among the most prevalent neoplastic diseases accounting for the highest mortality in both genders worldwide [1]. Due to typically asymptomatic early stage and late diagnosis, lung tumours are difficult to treat as the therapeutic options become limited. The reduction of lung cancer mortality has been set as a major priority in the UK through the development of more efficient early diagnosis and intervention tools, both of which require better understanding of the molecular biology of the disease[2].

Our previous studies suggested cytoglobin (CYGB) as a putative tumour suppressor gene in lung [3] and oesophageal [4] tumours and successive *in vitro* and *in vivo* studies provided further evidence in support of this hypothesis [5-7]. Nevertheless, CYGB was also associated with hypoxia and cancer aggressiveness [5, 8]. Shivapurkar and co-workers identified three downstream effector genes of CYGB, which were downregulated in CYGB-overexpressing lung and breast cancer cell lines [6]. These genes were: collagen 1A1 (COL1A1), RNA–binding and pre-mRNA Processing Factor (PRPF40A) and Uncoupling Protein 2 (UCP2). Interestingly, COL1A1, PRPF40A and UCP2 expression was linked to the metabolic pathways implicated in hypoxia and oxidative stress [9-12], events associated with more aggressive and therapy-resistant tumours [13, 14].

COL1A1 is an extracellular matrix protein, whose overexpression was linked to breast [15], gastric [16] and colorectal tumours [17]. UCP2 is a mitochondrial membrane protein transporting protons from the intermembrane space to mitochondrial matrix (i.e. uncoupling). Upregulation of UCP2 was reported in colon cancer [18], hepatocellular cancer, and cholangiocarcinoma [9]. Finally, PRPF40A participates in assembly of splicing machinery onto the pre-mRNAs [19]. Little is known about the involvement of PRPF40A in cancer.

However, its upregulation was reported in pancreatic cancer [20] and in the cells harbouring various p53 cancer-related mutations [21].

In previous microarray analyses, COL1A1 [22] and UCP2 [23] were found upregulated in various malignant tissues, including lung cancer. However, no single-gene validation of these results in lung cancer has been presented so far. Thus, we aimed to explore UCP2, PRPF40A and COL1A1 mRNA expression and promoter methylation in order to determine their molecular profile in NSCLC and their association to clinicopathological parameters and hypoxia markers.

Material and methods

Tissue collection

NSCLC and adjacent normal tissues were collected from 156 patients operated between 1995 and 2005 at the Liverpool Heart and Chest Hospital, UK. All patients submitted informed consent and the study was approved by the local ethics committee. The patient group [described in [24]] represents typical cohort with resectable lung tumour as decided following standard treatment procedures. It consisted of 99 males and 57 females, with the mean age 65.7 (range: 40 - 87). This set contained two most frequent histological subtypes: squamous cell lung cancer (SqCLC) (N=86) and adenocarcinoma (N=70). With regard to the pathological stage, tumours were split in the following groups: T1 (N=11), T2 (N=125), T3 (N=15) and T4 (N=5), while according to differentiation status the group breakdown was: poor (N=48) and moderate/good (N=107). Nodal metastasis occurred in 75 cases, while 80 patients had metastasis-free nodes. The follow-up data included 12 alive and 57 dead patients. The samples were snap frozen and grossly micro-dissected to ascertain

more than 80% tumour tissue. All the methylation and expression values were included in the analysis, even in the absence of normal, methylation or expression counterpart data for the given patient. Overall, we utilised RNA from 128 normal and 146 NSCLC tissues and DNA from 26 normal and 91 NSCLC tissues.

Cell lines

Adenocarcinoma (H358) and SqCLC (CALU1) lung cancer cell lines (ATCC) were maintained at 37°C/5% CO_2 in DMEM/F-12/Glutamax™-I (Invitrogen) with 10% FBS. For hypoxic studies the cells were cultured at 1% O_2 for 48h, while oxidative stress was achieved with 300μM H_2O_2 treatment for 24h. H_2O_2 concentration was chosen as an IC50 of CALU1 according to our survival curve.

Promoter methylation

DNA extraction, bisulfite conversion and pyrosequencing analyses were performed as described [24]. PCR reactions were performed with HotStarTaq®Plus Mastermix (Qiagen) according to manufacturer's protocol with 85ng bisulfite-converted DNA and 0.16μM primers. The following PCR primers (MWG Operon) were used: COL1A1_F GGAGAGAAGGTAAATGGAAG, COL1A1_R-biotinylated AACCTAACCCCAACCCTA, PRPF40A_F GAGTAGAGAATAGAGAGGATTTG, PRPF40A_R-biotinylated TAATCAAAACCCAAAAAAC, UCP2_F-biotinylated GTTTTGGGATTGATTGTT and UCP2_R CAAAACTAAAACCAAACTCAC. For pyrosequencing reaction 0.33μM sequencing primer was used: COL1A1_S

(GAGAAGGTAAATGGAAGA), PRPF40A_S (ATAGAGAGGATTTGGA) and UCP2_S (AAACTAAAACCAAACTC).

Gene expression

RNA isolation, reverse transcription, qPCR and data analysis were previously described [24]. The following primer/probe mixes were used in the qPCRs: COL1A1 (Hs01076780_g1), PRPF40A (Hs00215465_m1) and UCP2 (Hs01075225_m1) (Life Technologies).

GSH-Glow™ Glutathione Assay

Cellular level of glutathione was measured with the GSH-Glow™ Glutathione Assay (Promega) on Genios plate reader (Tecan) according to manufacturer's instructions. The luminescence values were normalised to the total protein level as assessed with the DC Biorad Assay (Biorad).

Statistical analysis

Non-parametric tests were used for statistical analyses (PASW Statistics 18.0, SPSS) as 1-sample Kolmogorov-Smirnov test showed skewed distribution of continuous variables. Pairwise comparisons between normal and NSCLC samples were performed with Wilcoxon test. The differences for continuous variables among independent cohorts within clinical parameters were determined with the Kruskal-Wallis and Mann-Whitney tests. Bivariate

correlation was probed with Spearman's test and Bonferroni correction was applied to adjust for multiple comparisons. Patients' survival was calculated with the log rank test.

Results

Gene expression analysis of COL1A1, PRPF40A and UCP2 in NSCLC

Our comparative qPCR analysis showed abundant COL1A1 overexpression in tumours (median RQ=975.4, IQR: 389.9-3172.7, N=132) when compared to adjacent normal tissues (69.4, 29.8-235.6, N=119, Mann-Whitney test, $p<1x10^{-4}$) (Fig.1A). Similarly, PRPF40A was overexpressed in NSCLC samples (RQ=3.49, 1.13-11.84, N=135 vs 1.53, 0.98-3.16, N=119, Mann-Whitney test, $p<1x10^{-4}$) (Fig.1B). In the case of UCP2, there was only a trend of higher expression observed in lung tumours (RQ=0.043, 0.025-0.08, N=136 vs 0.034, 0.026-0.055, N=122, Mann-Whitney test, p=0.066) (Fig.1C). UCP2 mRNA expression was higher in lung adenocarcinomas (RQ=0.053, 0.03-0.087, N=61) than in SqCLCs (RQ=0.035, 0.019-0.072, N=75, Mann-Whitney test, p=0.015). Apart from that no other associations were observed between COL1A1, PRPF40A or UCP2 mRNA expression and patients' gender, age, survival, smoking history, TNM classification, tumour histology and differentiation.

Promoter methylation analysis of COL1A1, PRPF40A and UCP2 in NSCLC

DNA for promoter methylation analysis was available for 91 of the NSCLC patients. We set the hypermethylation threshold according to a previously described method (Normal reference range = mean MtI + 2 x standard deviation of the normal samples) [24] at MtI=19.9% for UCP2 and MtI=13.0% for COL1A1. PRPF40A promoter was unmethylated in all samples

tested. UCP2 promoter hypermethylation was observed only in 5/91 NSCLC samples and none (0/26) of the normal tissues analysed. We noted elevated COL1A1 promoter methylation in 33/91 NSCLCs (Fig.1D) and in 2/26 normal samples. Although the difference in methylation between grouped normal and tumour samples was insignificant, pairwise comparison showed significant hypermethylation of COL1A1 promoter in NSCLC (p=0.035, Wilcoxon test). Higher methylation was observed in SqCLCs (median MtI=8.72%, IQR: 1.95-26.18, N=56) than in adenocarcinomas (2.28, 1.36-5.5, N=35, Mann-Whitney test, p=0.024, Fig.1E), as well as in the moderately and well differentiated tumours (MtI=5.19%, IQR: 1.99-28.21, N=65) in comparison to poorly differentiated NSCLCs (2.18, 1.39-5.45, N=26, Mann-Whitney test, p=0.01, Fig.1F). Apart from that observation COL1A1 methylation did not correlate with patients' gender, age, survival, smoking history and TNM classification. Moreover, methylation was not associated with the mRNA expression values, even when potentially confounding factors (histology, differentiation) were taken into account.

COL1A1, PRPF40A and UCP2 expression correlates with hypoxia markers

Next, we evaluated interrelationships between COL1A1, PRPF40A, UCP2 and hypoxia markers (CYGB, Hypoxia-Inducible Factor 1α - HIF1α and Vascular Endothelial Growth Factor - VEGFa), whose expression profiles were previously reported [24]. PRPF40A exhibited the strongest hypoxia association pattern among all genes under investigation (Table 1). Its mRNA expression level correlated with CYGB (ρ=0.7, $p<1\times10^{-4}$, N=128), HIF1α (ρ=0.841, $p<1\times10^{-4}$, N=129) and VEGFa (ρ=0.677, $p<1\times10^{-4}$, N=95). The hypoxia dependence was observed as well in the case of COL1A1, whose expression was associated with CYGB

(ρ=0.677, $p<1x10^{-4}$, N=128), HIF1α (ρ=0.646, $p<1x10^{-4}$, N=127) and, to a lesser extent, with VEGFa (ρ=0.356, $p=5.8x10^{-4}$, N=90). Similar relationships were seen in the case of UCP2 (UCP2 vs CYGB: ρ=0.38, $p<1x10^{-4}$, N=129, UCP2 vs HIF1α: ρ=0.596, $p<1x10^{-4}$, N=130 and vs VEGFa: ρ=0.343, $p=7.1x10^{-4}$, N=94). The expression profiles of COL1A1, PROPF40A and UCP2 genes correlated with each other. The strongest positive association was observed between PRPF40A and COL1A1 (ρ=0.612, $p<1x10^{-4}$, N=129), PRPF40A and UCP2 (ρ=0.596, $p<1x10^{-4}$, N=132), while the weakest – between COL1A1 and UCP2 (ρ=0.353, $p<1x10^{-4}$, N=128).

COL1A1, PRPF40A and UCP2 expression under stress conditions in vitro

Hypoxia response is activated not only with oxygen depletion, but also with nutrient deficiency, oxidative stress and other signalling pathways. Therefore, we wanted to assess whether COL1A1, PRPF40A and UCP2 might be regulated by hypoxia and/or oxidative stress *in vitro*. Cellular response to oxidative and hypoxic stress was confirmed by testing glutathione content (Fig.2A) and the mRNA expression level of VEGFa (Fig.2B), respectively. COL1A1 became upregulated in hypoxic conditions in both cell lines, however this was significant only in CALU1 (RQ=3.15±0.8 vs 1.0±0.07 in normoxic cells, p=0.02, Mann-Whitney), but not in H358 (RQ=2.2±0.7 vs 1.1±0.06, p=0.074). PRPF40A and UCP2 expression little changed at 1% O_2, as only CALU1 cell line exhibited modest upregulation of UCP2 (RQ=1.5±0,24 vs 0.9±0.15, p=0.04, Mann-Whitney). Similarly, oxidative stress did not evoke significant changes in the expression of COL1A1, UCP2 and PRPF40A genes.

Discussion

Although UCP2, PRPF40A and COL1A1 upregulation has been previously observed in malignant tissues [20, 22, 23] and associated to response to hypoxia and oxidative stress [9-12], little is known about their status in lung cancer. The data presented hereby demonstrate a trend of overexpression of UCP2 and significant overexpression of COL1A1 and PRPF40A in NSCLCs compared to adjacent normal tissues. Moreover, expression of all three genes correlated with that of hypoxia markers: CYGB, HIF1α and VEGFa. We also observed that PRPF40A promoter remained unmethylated, while UCP2 promoter was sporadically methylated in few cancer samples. Interestingly, we demonstrated a trend of increased methylation within COL1A1 promoter in a subset of NSCLCs, however, it did not associated with COL1A1 expression. This might be due to insufficient sample number taken into analysis, existence of another promoter controlling COL1A1 expression or presence of a potent factor that affects COL1A1 mRNA content more profoundly than DNA methylation, e.g. tumour hypoxia. Indeed, COL1A1 expression strongly correlates with hypoxia markers in tissues and becomes upregulated as well in low oxygen tension in our cell line model. Thus, the effect of hypoxia may be masking the methylation events observed in the subset of the NSCLC tissues. Surprisingly, our data indicate positive correlation of COL1A1, UCP2 and PRPF40A with CYGB expression, which has been previously shown to be inverse in *in vitro* experiments [6]. In the data published by Shivapurkar et al, transient CYGB overexpression resulted in >2-fold downregulation of the three genes, however this effect was less pronounced and reproducible in the cells with stable CYGB overexpression. While *in vivo* data not always recapitulate *in vitro* observations, prolonged tumour hypoxia identified in our tissue set may additionally modify the interdependence of CYGB and its proposed downstream effectors.

Despite the clustering of the three genes with hypoxia markers in tissues, *in vitro* hypoxia had differential effect on their expression. The statistical correlation between various genes might reflect similar, but unrelated expression patterns. These patterns could manifest the specificity of pathologic transcriptome rather than the activation of a particular signalling pathway. From this point of view, lack of responsiveness in the cells incubated at low O_2 level may indicate independence of PRPF40A and UCP2 from hypoxia in NSCLCs. Alternatively, the expression of PRPF40A and UCP2 may require additional stimulators, which are present in tumour microenvironment *in vivo*, but absent *in vitro* (e.g. more acute O_2 deficit, cytokine release, interactions with extracellular stroma) [25]. It is possible as well that in the lung cancer setting, UCP2 and PRPF40A act upstream of hypoxia pathways, thus inducing expression of HIF1α independently of the O_2 tension.

Whilst UCP2 overexpression might contribute to Warburg effect and promote tumourigenic phenotype [23], the implications of deregulated expression patterns of PRPF40A and COL1A1 remain to be elucidated. It is likely, however, that these genes may assist in the adaptive responses to oxidative stress and hypoxia / reoxygenation events, thus promoting tumour aggressiveness, metastasis and treatment resistance. As the three latter issues are pertinent to cancer patient management, it would of interest to investigate this hypothesis in the future.

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by gene expression profiling of lung adenocarcinoma xenografts identifies genes involved in tumor formation. *Mol Cancer Res* 2005;**3**: 119-29.

Table

Table 1. Association between mRNA expression levels of COL1A1, PRPF40A, UCP2, CYGB, HIF1α and VEGFa assessed with Spearman's correlation test. Rho-values (correlation coefficient), p-values and the number of samples for each analysis are provided. Each correlation appear significant after Bonferroni correction.

Table 1.

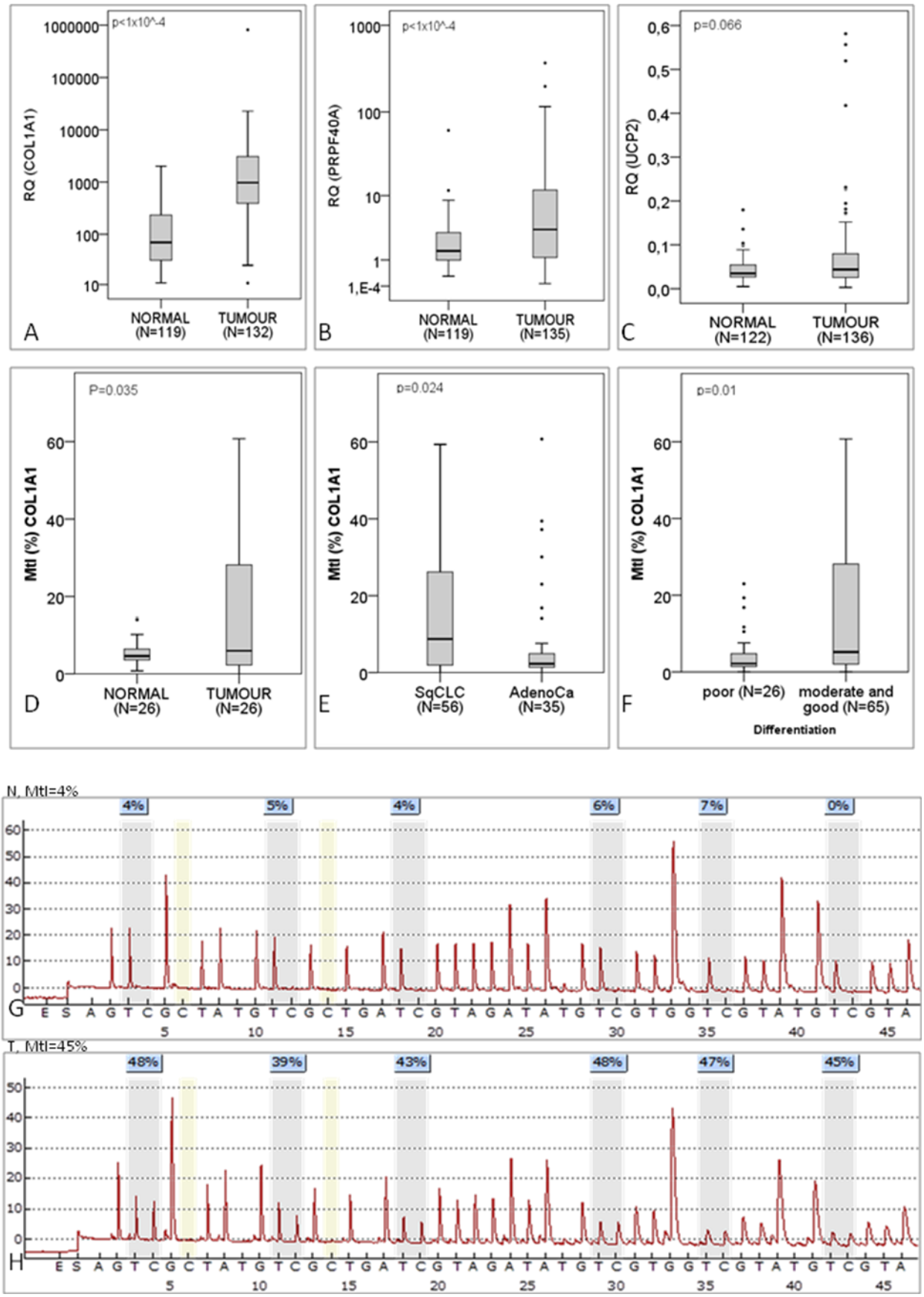
	PRPF40A	UCP2	CYGB	HIF1α	VEGFa
COL1A1	Rho=0.612, $p<1x10^{-4}$, N=129	Rho=0.353, $p<1x10^{-4}$, N=128	Rho=0.677, $p<1x10^{-4}$, N=128	Rho=0.646, $p<1x10^{-4}$, N=127	Rho=0.356, $p=5.8x10^{-4}$, N=90
PRPF40A		Rho=0.596, $p<1x10^{-4}$, N=132	Rho=0.7, $p<1x10^{-4}$, N=128	Rho=0.841, $p<1x10^{-4}$, N=129	Rho=0.677, $p<1x10^{-4}$, N=95
UCP2			Rho=0.38, $p<1x10^{-4}$, N=129	Rho=0.596, $p<1x10^{-4}$, N=130	Rho=0.343, $p=7.1x10^{-4}$, N=94

Figure legend

Figure 1. Boxplot representation of (A) COL1A1, (B) PRPF40A and (C) UCP2 mRNA levels in NSCLC and adjacent normal tissues. (D) *COL1A1* promoter methylation profile in NSCLC and normal samples, (E) in adenocarcinomas and SqCLCs, (F) and in the tumours with poor and moderate to good differentiation. Number of cases analysed with the Mann-Whitney (A-C, E, F) or Wilcoxon (D) test and corresponding p-values are provided. Representative pyrograms of COL1A1 promoter for normal (G) and NSCLC (H) samples analysed with pyrosequencing. X-axis indicates nucleotide dispensation order, while Y-axis luminescence intensity for each incorporated nucleotide. Boxes above the graphs show the percentage of methylated cytosines for each CpG site.

Figure 2. Glutathione (GSH) level normalised to total protein content (A) CALU1 and H358 cell lines after incubation with 300µM H_2O_2 (N=6). Relative mRNA expression of (B) VEGFa, (C) COL1A1, (D) PRPF40A and (E) UCP2 in CALU1 and H358 cell lines after incubation at 1% O_2 (H) or 300µM H_2O_2 (O). Columns represent mean (N=4) RQ values (±SEM) calibrated against untreated (N) control cells. * $p<0.05$ (Mann-Whitney test).

Figure 1.


p<1x10^-4
RQ (COL1A1)
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NORMAL (N=119)
TUMOUR (N=132)
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p<1x10^-4
RQ (PRPF40A)
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TUMOUR (N=135)
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RQ (UCP2)
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TUMOUR (N=136)
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MtI (%) COL1A1
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NORMAL (N=26)
TUMOUR (N=26)
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p=0.024
MtI (%) COL1A1
SqCLC (N=56)
AdenoCa (N=35)
E
p=0.01
MtI (%) COL1A1
poor (N=26)
moderate and good (N=65)
Differentiation
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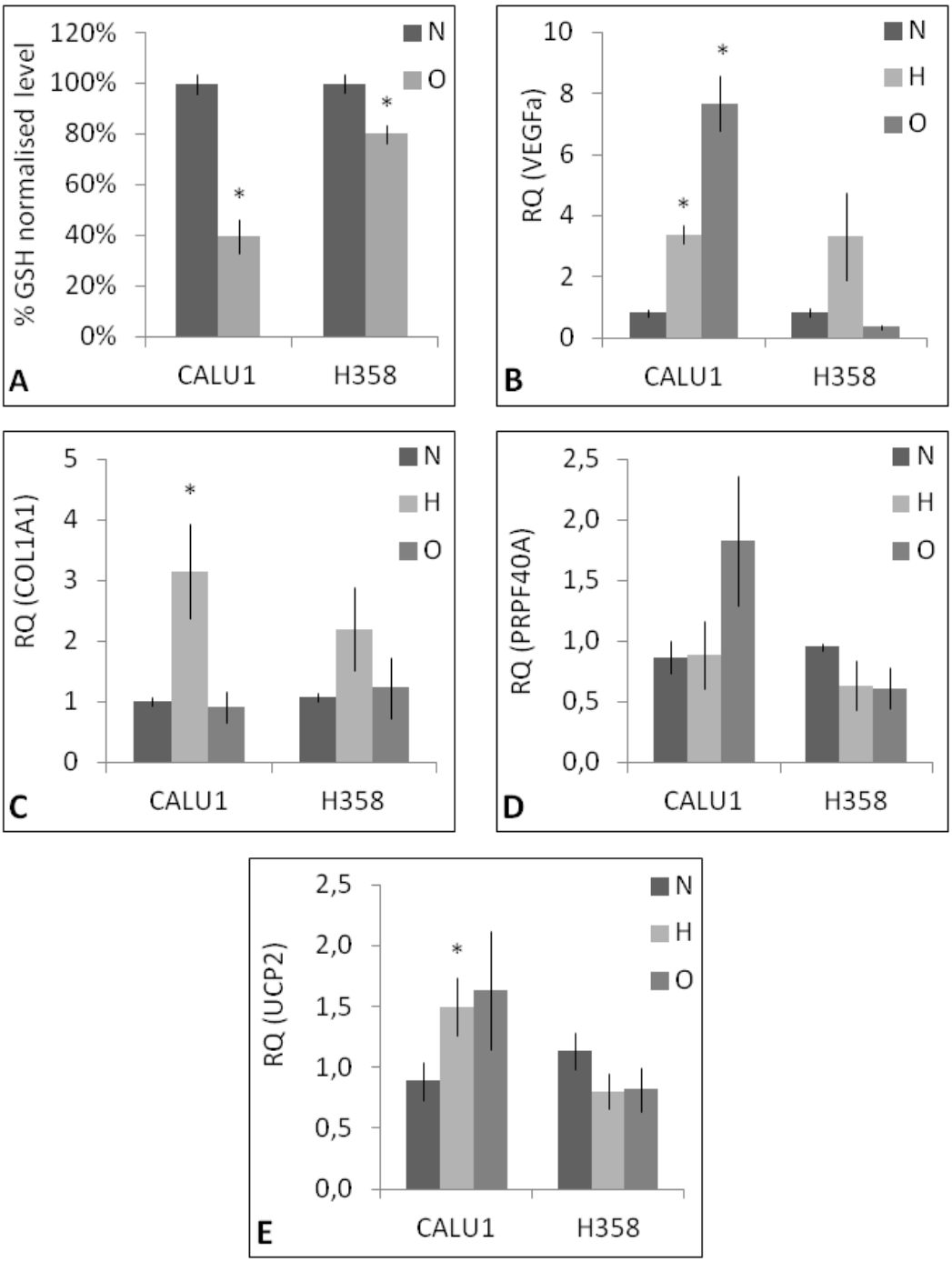
Figure 2.


% GSH normalised level
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