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IRP2 AS A POTENTIAL MODULATOR OF CELL PROLIFERATION, APOPTOSIS AND PROGNOSIS IN NON SMALL CELL LUNG CANCER

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Take home message: Iron loading causes cell proliferation in lung cancer. Iron chelation can return proliferation rates to baseline.

ABSTRACT

Background: *IREB2* is a gene that produces iron regulatory protein 2 (IRP2), which is critical to intracellular iron homeostasis, which relates to rate of cellular proliferation. *IREB2* lies in a lung cancer susceptibility locus.

Aims: To assess (i) Relationship between iron loading, cell proliferation and IRP2 expression in lung cancer (ii) Potential of iron related pathways as therapeutic targets (iii) Relevance of IRP2 in operated lung cancer patients.

Methods: Cells of two NSCLC lines and PBECS were cultured with and without iron; and proliferation, apoptosis and migration assessed. RT-PCR and Western blot were used to assess expression of iron homeostasis genes/proteins. Iron chelation and knockdown of *IREB2* were used *in vitro* to explore therapeutics. A cohort of operated NSCLC patients was studied for markers of systemic iron status, tumour IRP2 staining and survival.

Results: Iron loading caused cell proliferation in cancer cell lines, which were less able to regulate *IREB2* expression than PBECS. Iron chelation resulted in a return of proliferation rates to baseline levels; knockdown of *IREB2* had a similar effect. IRP2 positive tumours were larger ($p=0.045$) and higher percentage staining related to poorer survival ($p=0.079$).

Conclusions: Loss of iron regulation represents a poor prognostic marker in lung cancer.

INTRODUCTION

Lung cancer is the second most common cancer in the UK and is the leading cause of cancer mortality, accounting for 18% of all cancer-related deaths worldwide [1]. Cigarette smoke contains more than sixty carcinogens and there is convincing evidence that at least twenty of these cause tumour growth in lung tissue [2]. However, lung cancer is not a disease exclusive to smokers, as it has been estimated that 15% of cases in men and 53% of cases in women occur in people who have never smoked [3]. Despite improvements in screening [4] and surgical techniques survival remains poor and new avenues for therapy are urgently needed.

The largest genetic risk factor for lung cancer was thought to relate to tobacco smoking and subsequent nicotine addiction due to its locus within the nicotinic receptors (*CHRNA3-5*) [5]. However there is strong linkage disequilibrium (LD) between this and the gene coding for the iron regulatory protein IRP2 (*IREB2*), meaning that the true locus could lie within *IREB2*. Consistent with this, *IREB2* has been shown to be associated with COPD in genome wide association studies [6] and the expression of its protein product, IRP2, is altered in people with lung tumours who carry the lung cancer associated *CHRNA3-5* polymorphism [7]. The role of IRP2 is to control the level of cellular iron through the modulation of various iron import, export and storage proteins [8]. Therefore, faulty expression could cause a pro-tumorigenic change by altering levels of cellular iron, and deregulating iron import, export and storage proteins, thus allowing the development of malignant tumours through excessive iron loading within cells [9]. If IRP2 or iron pathways could be targeted therapeutically, it may provide a new modality in the treatment of lung cancer. Prior evidence that altered iron homeostasis can cause cellular proliferation in oesophageal and colorectal cancers, has informed the relevance of our hypotheses in the lung [10, 11].

We hypothesized that lung cancer cells would proliferate more rapidly in the presence of iron, and that this might be reduced by both iron chelation and alteration of *IREB2* expression.

Furthermore we hypothesized that tumours with altered IRP2 expression would relate to a change in clinical presentation and progression.

MATERIALS AND METHODS

Cell culture and iron stimulation

Experiments were performed in triplicate on two lung cancer cell lines (A549 and QG56) and primary bronchial epithelial cells (PBECS) using reagents purchased from Sigma-Aldrich, Dorset, UK. Cancer cells were cultured using RPMI-1640 with 10% FBS and 1% GPS and passaged when they reached confluence using 1x trypsin EDTA as described elsewhere [12]. PBECS were cultured using a BEGM BulletKit (Lonza Biologics plc, Slough, UK), which is serum-free. The RPMI medium contains no iron; the Lonza BEGM kit contains trace amounts of iron, that is $< 2\mu\text{M}$.

10mM FeSO_4 and 1mM sodium ascorbate solutions were filter sterilised and refrigerated for ≤ 7 days prior to serial dilution to the desired concentrations. Cells were trypsinised, counted and diluted, then left to adhere overnight prior to stimulation with iron loaded media for 24 hours. This was then replaced by iron free media for a further 24 hours at which point functional assays were carried out.

Assays of cell function, PCR and Western blotting

Proliferation was assessed by BrdU (Roche applied science, UK) according to the manufacturer's instructions, using 7×10^4 cells/ml. Migration was assessed using a scratch wound assay [13] with cells at 1×10^5 cells/ml; wounds were photographed and analysed using ImageJ software. Dual staining with annexin 5/propidium iodide fluorochromes (Invitrogen, Loughborough, UK) by flow cytometry was used to discriminate between apoptosis and

necrosis, as described previously [14]. Iron loading was assessed on cells at 1×10^5 cells/ml using Ferrozine, as described previously [15].

TaqMan gene expression assays assessed expression of *IREB2*, *TFRC*, *FTL*, *DMT1* and *HIF1a* which code for proteins involved in iron homeostasis (IRP2, transferrin receptor, ferritin, divalent metal transporter 1 and hypoxia inducible factor 1 alpha subunit). RNA was isolated using Trizol, converted to cDNA using RNA polymerase III, and used with appropriate housekeeping genes (*GAPDH* for *IREB2*, *TFRC*, *DMT1* and *HIF1a*, *18S* for *FTL*) in triplicate real time PCR experiments (all reagents Fisher Scientific, Loughborough, UK). Results are expressed as fold changes.

Protein was isolated from cells and measured using the Bio-Rad assay (Bio-rad Laboratories Ltd, Hertfordshire, UK). Westerns blots (WBs) were probed with IRP2 antibody (ab181153, Abcam, Cambridge, UK), Transferrin Receptor Monoclonal Antibody (13-6800, Fisher Scientific, Loughborough, UK), FTH1 (ab109373 Abcam, Cambridge, UK) and loading control beta actin (ab6276, Abcam, Cambridge, UK).

Similar experiments were performed (proliferation, apoptosis, necrosis, scratch wound assays and gene expression assays for *IREB2*, *FTH1* and *TFRC*) challenging the three cell lines with $150 \mu\text{mol}$ of Fe^{3+} . Methods and results from these experiments can be found in the Supplement.

Iron chelation

Stock solution of Deferasirox (10mM) was made, refrigerated in the dark, and serially diluted in sterile culture media to the working concentration when required. Chelation experiments were performed as above, returning cells to media containing deferasirox after iron incubation. Dose-response experiments were conducted as previously [16] and a dose of $150 \mu\text{M}$ of deferasirox chosen for further work.

IRP2 knockdown

SiRNA knockdown was achieved in cancer cell lines with Silencer Select siRNA against *IREB2*, using Silencer select negative siRNA as a control (both Fisher Scientific, Loughborough, UK), according to the manufacturer's instructions. Cells were incubated with the relevant siRNA on the day the cells were plated and then left for 24, 48 or 72 hours. The effect of iron exposure on knocked down cells incubated with the relevant siRNA for 48 hours was also explored.

Clinical relevance

Subjects

The clinical cohort has been described elsewhere [17]. Briefly, they comprise prospectively recruited patients who underwent pulmonary resection for lung cancer; those with tumour tissue available for histological work were included here (n=78). Pathological staging was conducted using latest staging guidance for non-small cell lung cancer (NSCLC) [18]. Circulating Hb, iron and ferritin levels were measured by routine clinical biochemistry and soluble transferrin receptor (sfTR) by ELISA (Abcam, Cambridge, UK). Survival was assessed using Cancer Intelligence data and GP records in September 2014. Pairs of normal and tumour lung from the same individual (n=35), with normal lung being distant to the tumour and microscopically uninvolved were used to describe the location of IRP2, and differences between tumour and health. The study was approved by the local ethics committee and all patients gave informed consent.

Immunohistochemistry

Lung samples were prepared as described previously [17] and stained for IRP2 using a protocol adapted from that published for fresh frozen lung tissue [6]. Staining involved a 30 minute CC1 antibody retrieval step, followed by 32 minutes antibody incubation (primary antibody anti-IRP2 LS-B675 1:1000 (Source Bioscience, Nottingham, UK)). Slides were scored by a pathologist using standard semi-quantitative techniques which grade intensity of staining

[19] against a positive control. IRP2 staining was deemed positive if >75% of the tumour had a stain intensity >0.

Statistical methods

All statistical analysis was carried out in SPSS 19.0 (IBM, USA). Paired t tests were used to compare in vitro conditions. Univariate tests for associations of IRP2 expression in vivo utilized the Chi square test and Mann-Whitney U tests according to the data type. Cox regression was used to assess impact of IRP2 expression on survival using, age and tumour stage as co-variables. Spearman's rank coefficient was used to assess correlations between IRP2 expression and variables such as histology and smoking. All tests were 2 tailed and significance assumed at $p < 0.05$.

RESULTS

Cell cultures with iron

Challenging all cell lines with 150uM FeSO₄ resulted in a significant increase in cellular iron loading (A549 and QG56, $p < 0.01$; PBEC $p < 0.05$) (Figure 1A). At the protein level in normal cells (PBECs) there was no change in IRP2, ferritin rose significantly and suppression of transferrin receptor occurred; in cancer cells no changes in IRP2 or transferrin occurred, and ferritin rose more markedly (Figure 1C). The expression of iron homeostasis proteins was generally lower in cancer cells than PBECs, as shown by the ratio to beta-actin. Cancer cell lines proliferated more in the presence of iron, unlike PBECs, with peak proliferation occurring at 150µM FeSO₄ (Figure 2A). The effects on proliferation were not seen with oxidized Fe³⁺ (Supplement). There were no significant differences in apoptosis, necrosis or migration with iron in any cell line (Figure 2B-D).

Chelation and IREB2 knockdown

Iron induced proliferation of cancer cells could be reduced below baseline levels when the chelator was used alone or alongside 150 μ M FeSO₄ (Figure 3). *IREB2* knockdown achieved 80% reduction in gene expression (Figure 4A); detailed time course experiments showed a concomitant change in IRP2 between 6 and 16 hours, which was maintained (Figure 4B). Reduced proliferation was seen in the presence of iron, similar to chelation (Figure 4C). Apoptosis did not differ with knockdown (mean (SEM) % apoptotic cells, A549 26.2 (6.2) v 15.8 (3.5); $p > 0.05$). Gene expression analysis of wild type and knocked down cells, when incubated with iron, showed a more marked *FTH* response and attenuated *TFR1* suppression (Figure 5). Wild type and knocked down cells, when incubated with iron, also showed increased expression of *DMT1* and *HIF1a* (Supplement).

Clinical data

Characteristics of the patients assessed for mortality are shown in Table 1. In normal lung IRP2 staining was evident in all samples on epithelial tissue and alveolar macrophages; tumours separated into those that were IRP2 positive, which constituted a minority of the sample and those that were negative (Table 1 and Figure 6). A graphical demonstration of IRP2 expression in each patient has been included in the Supplement. In univariate analyses to explore the determinants of positive IRP2 staining, higher stain intensity was seen in larger tumours (moderate or strong intensity: 31.7% (T1a and b) v 42.4% (T2a and b) v 100% (T4); $p = 0.045$, $\chi^2 = 12.9$). There was also a strong trend to a greater proportion of tumour being IRP2 positive in patients who died (27.5% v 10.0% of tumour; $p = 0.079$). Cox regression analyses showed that age ($p < 0.0001$) and tumour stage associated with survival ($p = 0.021$; odds of death for stage 1a/b patients = 0.23 (0.07-0.81), but positive IRP2 staining did not ($p = 0.31$). There was no relationship observed between positive IRP2 staining and histology ($r_s = -0.035$, $p = 0.785$) or between positive IRP2 staining and smoking status ($r_s = -0.048$, $p = 0.711$) or pack year history ($r_s = -0.049$, $p = 0.784$). Further analyses correlating serum levels of haemoglobin, ferritin, iron

and transferrin against histology, smoking status and pack year history can be found in the Supplement.

| | |
|------------------------------------|----------------|
| Age | 69.4 (0.97) |
| Male gender | 57 (73.1%) |
| Pack years smoked | 20 (15-150) |
| Current smoker | 26 (33.3%) |
| Never smoked | 2 (2.6%) |
| Tumour stage | |
| 1a | 18 (23%) |
| 1b | 25 (32%) |
| 2a | 14 (18%) |
| 2b | 3 (4%) |
| 3a | 17 (22%) |
| 3b | 1 (1%) |
| Cancer death | 17 (21.8%) |
| Other cause of death | 23 (29.5%) |
| Years of follow up | 7.36 (3.2-9.7) |
| Hb (g/l) | 12.7 (0.4) |
| Tumour stage 1 | 12.39 (0.27) |
| Tumour stage 2 | 13.79 (0.47) |
| Tumour stage 3 | 11.9 (0.53) |
| Tumour stage 4 | 12.43 (0.36) |
| Ferritin | 134.05 (13.09) |
| Iron ($\mu\text{mol/l}$) | 9.86 (1.18) |
| sTfR (mg/l) | 2.99 (0.07) |
| IRP2 positive tumour | 18 (23.1%) |
| IRP2 stain intensity | |
| Low | 34 (43.6%) |
| Medium | 20 (25.6%) |
| High | 4 (5.1%) |
| IRP2 percentage of tumour positive | 20 (1-100) |

Table 1: Characteristics of the patients

Data is shown as mean (SEM) or median (range) if non-normally distributed. Frequency variables are shown in italics and are denoted n (%).

None of the markers of systemic iron homeostasis showed any relationship to tumour size or survival (all $p > 0.51$). No clear relationships were seen between systemic iron markers and tumour IRP2 (all $p > 0.61$).

DISCUSSION

This study has shown that abnormal iron-mediated cell proliferation can occur in lung cancer, and that this is mediated significantly by *IREB2*. This may be amenable to therapy, and clinically important, as demonstrated by the differences in IRP2 staining between tumour and health, and the suggestive relationship of IRP2 staining to tumour size and survival respectively.

Iron mediated cancer cell proliferation

Our data shows that lung cancer grows more rapidly in the presence of iron, an effect which is far less marked in normal cells (PBECs). Several neoplastic diseases have been linked with iron homeostasis abnormalities. For example, high hepatocellular carcinoma rates are seen in hereditary hemochromatosis, where iron accumulates in the liver; this has been attributed to the increased iron levels [20]. Furthermore, in colorectal cancer an increase in the levels of intracellular iron within cancer cell lines caused a corresponding increase in cellular proliferation [10]. A similar phenomenon was observed in oesophageal adenocarcinoma [11].

There are a number of different reasons why accumulation of iron within tumour cells may drive hyper-proliferation. The first is through an increase in Wnt signaling, a major oncogenic signalling pathway, especially within epithelially derived tumours [21]. Wnt signalling drives cellular proliferation due to accumulation of beta catenin-transcription factor (TCF/LEF) complexes, which cause activation of target genes such as *c-myc* [22]. These pathways, rather than E-cadherin dependent ones, seem to be most relevant to iron mediated Wnt signaling in cancer and might be driven by acquired mutations in cancer cells [23]. Alternatively iron might mediate tumorigenesis by oxidative stress, whereby oxygen radicals are able to damage cellular DNA, leading to mutations within tumour suppressor genes, oncogenes, or enhancement of the effect of pre-existing mutations [24]. Thirdly iron may influence ribonucleotide reductase activity, the rate limiting step in DNA synthesis. If there is an increase in cellular iron, there is a consequent increase in ribonucleotide reductase activity and so a greater rate of DNA synthesis,

cellular proliferation and cancer [25]. The final mechanism of iron driven hyper-proliferation is through increased activity of the iron sensitive phosphatase CDC14A, which is directly involved in the regulation of cell cycle checkpoints through its interactions with P53. Increases in cellular iron drive the phosphatase to progress the cell through checkpoints faster and limits forced arrest and apoptosis, thus causing or exacerbating cancer [26, 27]. Iron loading did not appear to affect apoptosis in our study, however the production of free radicals due to iron could explain the trend to increased levels of necrosis in PBECs as hydroxyl radicals can be injurious to cells in multiple ways.

The gene expression studies were intended to give further insight into possible dysregulation of iron homeostasis genes in lung cancer. We observed an increase in *IREB2* in iron exposed cells, which would translate to a rise in unbound IRP2, however this was barely seen in our WBs. This is most likely because in iron replete, normoxic cells IRP2 usually undergoes degradation via the proteasome [28]. In iron replete conditions IRP2 does not bind to IREs; binding stabilizes *TFRC* mRNA, thus in the absence of IRP2 binding low *TFRC* levels would be expected, as was observed in normal PBECs. In the cancer cell lines this did not occur to such an extent, indeed not at all in our squamous line (QG56). Interestingly *IREB2* knockdown did not affect *TFRC* expression in our lines; if IRP2 were the main driver of *TFRC* stability (and therefore levels) in these cells we would have expected to see this. *TFRC* can be affected by oncogenes [29], which might well be influencing the cancer lines, and abrogating the effect of loss of *IREB2*. We also observed a rise in ferritin in cancer cell lines, which was less marked in normal PBECs. In a cell with increased levels of iron, ferritin would be expected to increase to bind the excess iron and *TFRC* activity is reduced to prevent further iron uptake [30]. Since the cancer cell lines were not regulating *TFRC*, iron entry continued unimpeded and a rise in *FTH* was required to compensate. Conversely in normal cells iron entry was suppressed by reduction of *TFRC*, so a lesser increase in ferritin was required to regulate intracellular iron concentrations. Of note, our cells took up iron in the Fe²⁺ form, which is unusual when looking at the wider literature. As a

result, we hypothesise that there is a novel mechanism at play here, perhaps due to the use of a synthetic compound like sodium ascorbate to oxidise our Fe²⁺ to Fe³⁺.

Results consistent with our findings regarding proliferation, iron loading and iron homeostasis genes were reported in colorectal cancer, where an increase in the expression of *TfR* coupled with a decrease in the expression of ferroportin led to iron loading within gastric epithelium and the formation of adenocarcinomas [10]. A similar phenomenon was seen in oesophageal adenocarcinoma; although there was an increase in iron export machinery (ferroportin) the protein was cytoplasmic, implying a lack of functionality [11].

Clinical data

The immunohistochemistry showed that only a small proportion of lung cancers exhibit IRP2 staining. Since IRP2 would usually be degraded in normoxic, iron replete cells [28] continued expression in the context of a tumour could reflect a response to counter local hypoxia or iron starvation due to rapid tumour growth. Consistent with this, IRP2 positive tumours tended to be larger and survival rates were poorer; this result is consistent with breast cancer, where increased IRP2 expression is seen in higher grade tumours [31]. However the survival effect was not maintained in multivariate analysis, possibly due to the known relationship between tumour size/stage and survival, which may have confounded IRP2 survival analyses. Whether the subset of tumours that are IRP2 positive represent a cohort of patients with an acquired mutation in their tumour, as has been suggested in colorectal cancers that fail to regulate iron homeostasis [32], or whether they come from patients with an innate genetic susceptibility to lung cancer was not possible to assess with the available samples.

Potential for therapy

The iron chelation experiments showed that use of an orally administered iron chelator could reduce proliferation of cancer cells, albeit also with a greater effect on normal cells (PBECS). This is broadly consistent with the effects seen by others [16], and in cancers outside the lung

[10, 33]. The mechanism of action of deferasirox is two fold, firstly removal of iron from cells by the formation of intracellular complexes of existing iron and the subsequent prevention of uptake of new transferrin bound iron [20]. There is also a direct effect on tumour cells whereby deferasirox significantly up-regulates the metastasis suppressor *NDRG1* which then up-regulates p21 [34] and significantly decreases cyclin D1 [35]. This causes the cell cycle to arrest at the G₁/S checkpoint, leading to decreased proliferation [36].

In addition to potential local effects on healthy lung tissue, iron chelation has the potential to cause significant side effects such as cardiac fibrosis and anaemia [37]. Our cohort did not exhibit significant anaemia, even in the advanced stages of cancer, but it would be wise to check the baseline haemoglobin and monitor this carefully if iron chelation were used clinically. We hypothesized that a different driver of local iron regulation, such as IRP2, might represent an equally good therapeutic target for reduction of intracellular iron that does not suffer from these limitations and is relevant to lung cancer. IRP2 induction has increased tumour growth in a mouse xenograft model, suggesting it to be a reasonable target [9]. The data was supportive of this, showing a similar reduction in cellular proliferation with knockdown of *IREB2* in cancer cells as was seen with desferasirox. Similar results have been seen in breast cancer [31]. Inhaled RNAi has recently been developed in a novel form and used in mice against different genetic targets to treat lung cancer [38], implying that this might be a potential way to develop *IREB2* modulation as a therapy in NSCLC. We did not observe any relationships between outcome and systemic iron status; this could imply that only local (i.e. airway) iron is of relevance, as luminal iron appears to be of most relevance to colorectal cancer [32], but the multiple influences on systemic iron parameters make interpretation of this data less clear.

Limitations and further work

Our study is limited to surgically resected cases of NSCLC which led to relatively small numbers for the survival analyses; nevertheless the cohort remains competitive in the field for its size and degree of characterization. The proportion of female patients is relatively high, and

many cases were quite advanced on pathological staging (stage IIIa or b) which could limit generalizability. We did not formally account for adjuvant therapy use in our analyses, since only 3 patients received it, but acknowledge that there is a small chance this could affect results, for example cisplatin can manipulate iron deposits during chemotherapy [39]. Perl's staining to measure non-heme iron was not conducted because of its low sensitivity and difficulty in reproduction due to the fact that smokers' pigment in alveolar macrophages is Perl's positive [40]. Our Supplement shows that our findings are not reproducible under conditions of Fe³⁺. The lack of effect we have seen with Fe³⁺ is interesting, because iron is presumed to be oxidised within the airway lumen, but we do not know how important this is in the grand scheme of things: greater emphasis may need to be placed on iron levels within the tissue and blood.

Further work might include assessment of airway lumen iron concentrations, which varies in smokers [41], and local levels of neutrophil elastase, which has been shown previously to influence iron concentrations in bronchoalveolar lavage fluid [42]. This would be useful to assess if local rather than systemic chelation treatment has any potential for benefit, and if so whether smokers or patients with COPD might be more likely to benefit. Other mechanisms that could be explored in future work include mitochondrial dysfunction and ferroptosis. Recent animal work has shown IRP2 to promote mitochondrial dysfunction and iron overload in COPD [43]. This suggests a likely role for mitochondrial chelators in the treatment of COPD, and could further be explored in animal models of lung cancer. Animal models would also be indispensable for studying the side effects of iron chelators in cancer. Ferroptosis is a recently characterized form of cell death, distinct from apoptosis and necrosis, where mitochondria are smaller than normal, with reduced crista and outer membrane rupture [44]. Iron overload contributes to ferroptosis through production of reactive oxygen species. Conversely, inhibition of iron uptake prevents erastin-induced ferroptosis [45]. Whilst kidney and leukaemic cell lines appear to be most sensitive to erastin [46], further study of role of erastin-induced ferroptosis in lung cancer cells exposed to iron chelators could be of interest in creating an opportunity for therapeutic intervention.

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