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RESEARCH

Expressions of Topoisomerase IIα and BCRP in Metastatic Cells are Associated with Overall Survival in Small Cell Lung Cancer Patients

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Abstract The aim of this study was to investigate the mRNA expression levels of multidrug resistance-associated proteins in chemo-naïve metastatic lung cancer cells and to determine the correlation with response to chemotherapy and overall survival. Metastatic cells were obtained by transbronchial fine needle aspiration biopsy of enlarged mediastinal lymph nodes in 14 patients with small cell lung cancer (SCLC) and 7 patients with non-small cell lung cancer (NSCLC). After cytological confirmation of lung cancer type, total RNA was extracted from biopsy samples and reverse transcribed to cDNA, and real-time PCR for the genes of interest [P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1), breast cancer resistance protein (BCRP), lung resistance protein (LRP) and topoisomerase IIα (TOPIIα)], was performed. We observed significantly decreased expression of BCRP and significantly increased expression of TOPIIα in metastatic SCLC cells compared to NSCLC. Furthermore, in SCLC high topoisomerase IIα and low BCRP expression levels positively correlated with longer overall survival. Our results showed higher expression levels of BCRP as well as lower levels of topoisomerase IIα in chemo-naïve metastatic cells in NSCLC than in SCLC. These results correlate with previous observations that metastatic SCLC cells at the beginning of chemotherapy are potentially more sensitive to chemotherapeutic agents while in metastatic NSCLC cells resistance is usually inherent. We also showed that altered levels of topoisomerase IIα and BCRP in SCLC are important factors that contribute to resistance to chemotherapeutics that interfere with the enzyme and/or DNA and are highly associated with overall survival.

Keywords Small cell lung cancer · Metastatic cells · Topoisomerase IIα · Breast cancer resistance protein (BCRP) · mRNA expression levels

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Introduction

Despite recent advantages in the management of early disease, lung cancer remains an important clinical problem. It is the most commonly diagnosed cancer and the most common cause of cancer death worldwide. Chemotherapy is primary treatment for small cell lung cancer (SCLC) and is increasingly used in non-small cell lung cancer (NSCLC).

The use of chemotherapy has clearly been shown to provide survival benefits to several different patient groups and cancer types [1, 2]. However, resistance to chemotherapy is one of the most important therapeutic burdens for successful lung cancer therapy; therefore, a better understanding of drug resistance mechanisms is needed to improve survival in this group of patients. Some tumours are initially resistant and never respond to cytostatic drug treatment but others often relapse after a good initial response [3]. In SCLC, resistance is usually associated with the emergence of drug-resistant cell clones during chemotherapy [4] and NSCLC often shows intrinsic multi-drug resistance [3, 5–7]. Therefore, the

expression levels of molecules conferring resistance should differ between SCLC and NSCLC. Those differences were confirmed in primary tumour cells but little is known about metastatic cells that are the real cause of treatment failure and disease progress. The major mechanisms involved in the multi-drug resistance to chemotherapy of cancer cells are up-regulation of resistance molecules or down-regulation of target molecules [3, 8]. The first mechanism that could contribute to several structural and functional unrelated cytotoxic agents, such as epipodophyllotoxins, *Vinca* alkaloids, anthracyclines, taxanes, colchicines and others, is associated with altered cytotoxic drug transport that is mediated by members of the ABC superfamily of transport proteins. The most well known are P-glycoprotein (P-gp, also known as MDR1), Multidrug resistance protein 1 (MRP1) and Breast cancer resistance protein (BCRP) [9–13]. Besides the overall reduction of intracellular drug concentration, redistribution of the drug to cytoplasmic vesicles has been employed in drug resistant cancer cells. Lung resistance protein (LRP), which is an integral part of the major vault protein and is found in the cytoplasm and nuclear membrane, is involved in the intracellular distribution of cytotoxic agents [13–15]. Several chemotherapeutic agents involved in treating cancer interfere with topoisomerase IIα activity, such as anthracyclines and etoposide, or microtubule stabilisation, such as taxanes [8]. Therefore, another mechanism of drug resistance is associated with altered expression of the target (e.g., topoisomerase IIα). Accordingly, the higher the concentration of topoisomerase IIα, the better the effect of the cytotoxic agent and consequently better response and better survival [8, 16–18].

Because the characteristics of various lung cancers differ between individuals, several different strategies are used to improve the prognosis of lung cancer, varying in their durations, toxicities and costs. Ideally, chemotherapy should be customised to each individual patient based on the nature of their particular stage and disease. Detection of one or a combination of different predictive markers would allow us to select the effective individual therapies and it would also help us to avoid unnecessary treatments. Therefore, in some very responsive tumours, shorter and less-toxic regimens would be sufficient while others may require more intensive therapy, a longer duration or different anticancer drugs [19].

This study focused on chemo-naïve metastatic lung cancer cells from mediastinal lymph nodes. The aim was to investigate the levels of P-gp, MRP1, BCRP, LRP and topoisomerase IIα mRNA expression at diagnosis in chemo-naïve patients and their correlation to the overall response to chemotherapy and survival rate in patients with SCLC and NSCLC in metastatic mediastinal lymph nodes.

Materials and Methods

Patient Selection

We initiated a prospective study after it was approved by the state ethical committee and after patients gave their informed consent. The study was focused on patients with WHO/ECOG performance status 0 or 1 and disease stage of IIIB or IV with clearly positive and morphologically evident SCLC or NSCLC (adenocarcinoma or squamous cell lung carcinoma) in metastatic cells obtained from mediastinal lymph nodes and who had no prior chemotherapy or radiotherapy. Samples for cytological examination and RNA analysis were obtained by transbronchial fine needle aspiration biopsy (TBNA) of mediastinal lymph nodes under endobronchial ultrasound (EBUS) guidance. We excluded all patients with lung cancer whose mediastinal lymph nodes were not invaded with tumour cells, samples from enlarged lymph nodes that were not diagnostic or samples which contained lymphocytes and/or respiratory epithelial cells. After diagnosis was confirmed, all SCLC patients received cisplatin/etoposide and/or cyclophosphamide/epirubicin (doxorubicin)/vincristine. Due to small number of NSCLC patients, no further analyses were performed in chemotherapy response studies.

The response rate to chemotherapy was evaluated after completion of the fourth cycle of chemotherapy by response evaluation criteria in solid tumours (RECIST criteria). The overall survival was the time from the diagnosis of the cancer to the death of the patient or date of last visit.

We compared the levels of P-gp, MRP1, BCRP, LRP and topoisomerase IIα expression in metastatic cells of different lung cancer types and in different disease stages.

RNA Extraction and Reverse Transcription

Biopsy samples were stored at −40°C in RNA*later* Solution (Ambion Inc., Austin, TX, USA) until isolation procedures were performed. Total RNA was extracted using a RNeasy Mini Kit and QIAshredder in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). The quantity and quality of the isolated RNA was determined by measurement of the absorbance at 260 and 280 nm and with Qubit (Invitrogen Corporation, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The reverse transcription reaction was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The cDNA was stored at −40°C.

Quantitative Gene Expression Analysis

Real-time PCR was performed on an ABI PRISM 7500 Real-Time PCR System (Sequence Detection System instrument equipped with SDS version 1.3.0 software) (Applied Biosystems, Foster City, CA, USA). The ribosomal 18S RNA was used as an endogenous control for normalisation of the target genes. Primers and probes for the 18S rRNA were supplied as a PDAR (predeveloped assay reagent) from Applied Biosystems with the probe labelled with VIC at the 5′ end. Primers and probes for the genes of interest (ATP-binding cassette, sub-family B (P-gp), ATP-binding cassette, sub-family C (MRP1), ATP-binding cassette, sub-family G (BCRP), major vault protein (LRP) and topoisomerase II α (TOPIIα)**)** were also supplied from Applied Biosystems as TaqMan® Gene Expression Assays with the MGB probes labelled with FAM at the 5′ end (assays IDs are Hs00184491_m1, Hs00219905_m1, Hs01053787_m1, Hs00911181_m1 and Hs00172214_m1, respectively).

PCR reactions were set up in separate tubes with TaqMan Universal PCR Master Mix (Applied Biosystems) at default thermal conditions: 2 min at 50°C, 10 min at 95° C for enzyme activation and then 45 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. All measurements were performed in triplicate for each time point and relative expression was analysed using the ΔΔCt method. Briefly, the relative expression of each mRNA was calculated by subtracting the Ct value of ribosomal 18S RNA from the Ct value of the target mRNA to calculate the ΔCt. Then this ΔCt value was compared with the ΔCt value of the control tissue. This study used the resected mediastinal lymph nodes from a control subject as the calibrator.

Statistical Analyses

The distribution of data was recalculated by the Shapiro-Wilk normality test. The strength of association between the expression levels of the selected genes and other patients and tumour characteristics was tested with the Unpaired *t* test or the Mann–Whitney *U* test. Correlation between overall survival with expression profiles was calculated using the Kaplan-Meier method. Probability values of $P<0.05$ were accepted as significant. Analyses were performed with GraphPad Prism 5.

Results

A total of 21 patients, 14 with small cell and 7 with non-small cell lung carcinoma [4 women, 17 men; median age 65 years (range: 53–78)] were included in this study. The mRNA expression levels of P-gp, MRP1, BCRP, LRP and TOPIIα, estimated by real-time RT-PCR, were expressed in arbitrary units [($\log_{10}$ (Relative Quantitation)] for all SCLC and NSCLC metastatic tumour cells.

First, we investigated the expression of these genes in relation to different lung cancer cell types. We observed differences in the patterns of expression between SCLC and NSCLC in metastatic cells. Median mRNA levels [$\log_{10}$ (Relative Quantitation)] for BCRP (0.780 vs. –0.227; $P=0.013$; Unpaired *t* test) were significantly higher in the NSCLC samples than in the SCLC samples while TOPIIα mRNA levels were higher in the SCLC samples than in NSCLC samples (1.083 vs. 0.295; $P=0.002$; Unpaired *t* test). P-gp, MRP1 and LRP mRNA levels did not differ significantly between those two types of lung cancer, however MRP1 and LRP mRNA levels tended to be higher in the NSCLC samples (Fig. 1).

Subsequently, we compared the mRNA expression levels of P-gp, MRP1, BCRP, LRP and TOPIIα in metastatic SCLC cells in chemo-naïve patients and compared that to the chemotherapy response according to RECIST criteria. The response rate to chemotherapy showed progressive disease (PD) in 1 patient, stable disease (SD) in 1 patient, partial response (PR) in 7 patients, complete response (CR) in 4 patients and we were unable to determine the response rate in 1 patient (NA). Hence, 12 patients showed an objective response (CR, PR or SD) while 1 patient was classified as a nonresponder (PD). Among patients with an objective response, those with better responses (CR and PR) had higher TOPIIα mRNA expression levels. All patients with CR and 5 with PR had high TOPIIα (higher than 1) levels while all patients with SD and PD had low TOPIIα levels. There was no clear correlation between mRNA expression levels and response rate to chemotherapy for other markers.

When we compared SCLC patients with different stages of disease (i.e. 7 with limited-stage disease (LD) and 7 with extensive-stage disease (ED)) we found that patients with LD had much longer overall survival compared to patients with ED. The ratio for longer overall survival for LD compared to ED was 1.81 (95 Cl%, 1.46–2.17; $P<0.001$). Furthermore, all patients with LD had high mRNA expression levels of TOPIIα while in the patients with ED, only 3 patients had high mRNA expression levels, and 4 had low expression. A difference was also observed for BCRP mRNA expression levels where there were 2 patients with LD that had high levels (higher than −0.3) and 5 patients with low levels of BCRP while in patients with ED, 6 had high BCRP mRNA expression levels, and only 1 patient had low levels of BCRP. Other expression profiles did not differ between those two stages.

The expression levels of the drug resistance genes were further analysed in relation to overall survival. Kaplan-

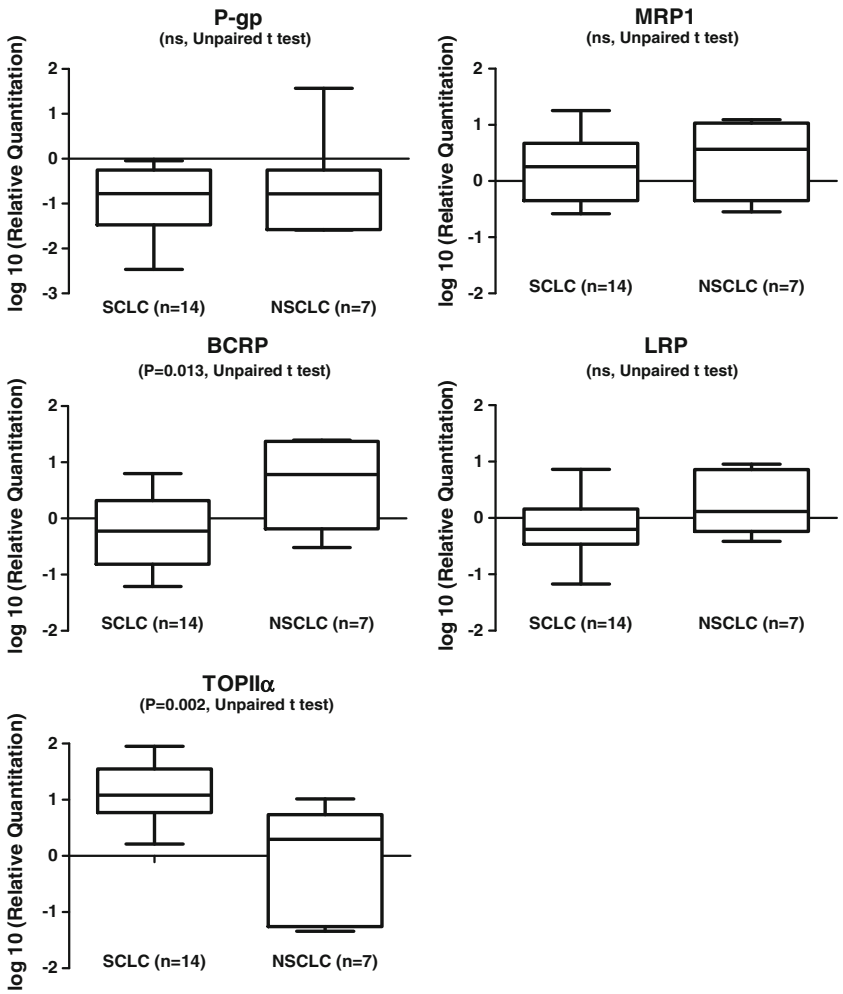

Fig. 1 Comparison of relative mRNA expression levels between SCLC and NSCLC metastatic cells for selected genes (P-gp, MRP1, BCRP, LRP and TOPIIα). Box plots with whiskers from minimum to maximum. The line in the middle represents the median

Meier curves stratified by expression of respective drug resistance genes as low vs. high revealed that P-gp, MRP1 and LRP expression did not significantly relate to the length of overall survival in SCLC patients. However, low expression levels of TOPIIα were highly associated with poor overall survival (Fig. 2). The ratio for low expression (as compared to high) of TOPIIα mRNA, was 1.79 (95% Cl, 1.47–2.10; P=0.008). On the other hand, high expression levels of BCRP were associated with poor overall survival (Fig. 2). The ratio for low expression (as compared to high) of BCRP mRNA, was 0.61 (95% Cl 0.27–0.96; P=0.034).

Discussion

In this study we evaluate the mRNA expression levels of P-gp, MRP1, BCRP, LRP and topoisomerase IIα in SCLC and NSCLC metastatic cells. To our knowledge, this is the first study to compare the mRNA expression levels of those

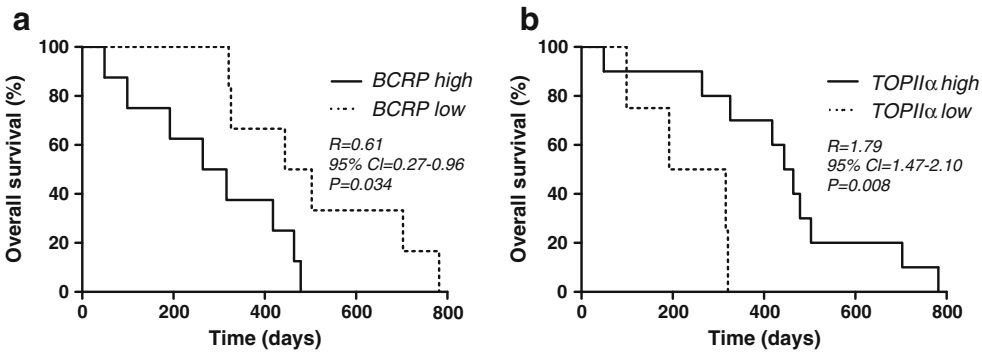

Fig. 2 Kaplan-Meier curves for overall survival as a function of the relative mRNA levels of BCRP (**a**) and TOPIIα (**b**) in SCLC patients

markers in SCLC and NSCLC in chemo-naïve metastatic cells to the benefits of chemotherapy, response to cytotoxic agents and overall survival. The mediastinal lymph node sampling also helped us overcome the errors contributed to the contamination of tumour tissue with epithelial tissue and lymphocytes because it has been previously reported that ABC transporters along with topoisomerase IIα are also expressed in healthy tissue [8, 20].

Our results indicate that basal levels in chemo-naïve metastatic cells (before the start of the chemotherapy) of BCRP are higher in NSCLC while topoisomerase IIα levels are much higher in SCLC. This is in agreement with previous findings from primary tumour cells, which stated that in SCLC, response to chemotherapy is initially successful, but it often relapses after acquired resistance, and changes in expression levels of multi-drug associated mechanisms occur while in NSCLC resistance is inherent [3–8, 13, 21]. Several previous studies have indicated that a novel transport protein of the ABC superfamily (BCRP) is involved in multi-drug resistance by pumping out the cytotoxic agent, therefore conferring resistance [9–12, 21–23]. Additionally in some recent reports it has been shown that altered levels of the target molecule, in this case topoisomerase IIα, is an important factor in the multi-drug resistance phenotype [3, 8]. This is in agreement with our finding because there are much higher levels of topoisomerase IIα in SCLC, which is sensitive to chemotherapeutic agents at the beginning of the treatment, while NSCLC generally has inherent resistance.

Furthermore, in SCLC patients, high expression levels of topoisomerase IIα were also in good correlation with longer overall survival. This is in clear agreement with the findings that higher levels of topoisomerase IIα are a good predictor of responses to chemotherapeutic agents and survival [8, 16].

Apart from topoisomerase IIα expression, the drug resistance gene BCRP had additional predictive value for response to chemotherapy because low mRNA expression levels of this transporter correlated with a longer overall survival of SCLC patients. No associations between expression levels and response or overall survival were determined for the other markers.

In summary, the results of this study in metastatic cells support the previous findings of different expression levels of multi-drug associated protein BCRP as well as topoisomerase IIα in primary tumour cells between SCLC and NSCLC before the start of chemotherapy, which is in consensus with different cancer characteristics and an initial response to cytotoxic agents. In addition, we showed that in chemo-naïve metastatic cells altered mRNA expression levels of topoisomerase IIα as well as BCRP strongly correlates with the length of overall survival among the selected SCLC patients that underwent chemotherapy. The improvement in molecular as well as in other diagnostic techniques in the near future will allow us to tailor chemotherapy to each individual patient according to their particular stage and disease. Topoisomerase and BCRP appear to be good markers for determining the outcome of the treatment and length of overall survival. Based on the expression levels of the enzyme and the transporter, appropriate therapy regimens that are better suited to each individual could be selected. However, further investigations, with more patients and studies on relapsed disease are needed to confirm the findings of this study.

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Conflict of Interest Statement We declare that we have no conflict of interest.

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