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Decreased expression of cytochrome p450 1B1 in non-small cell lung cancer

Bartosz Kazimierz Słowikowski[a,*], Bartłomiej Gałęcki[b], Wojciech Dyszkiewicz[b], Paweł Piotr Jagodziński[a]

[a] Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, Święcickiego 6 Street, 60-781 Poznan, Poland
[b] Department of Thoracic Surgery, Poznan University of Medical Sciences, Szamarzewskiego 62 Street, 60-569 Poznan, Poland

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ABSTRACT

Recent studies have associated oestrogen metabolism and cigarette smoking with their carcinogenic impact on the lungs. Compounds commonly found in tobacco smoke induce the activity of CYP1B1, the enzyme responsible for the synthesis of catecholic derivatives of oestrogens. During their redox transformations, these structures can release large amounts of reactive oxygen species or can form DNA adducts, which lead to the decomposition of genetic material. This process may illustrate the synergistic effect of oestrogenic activity and tobacco combustion on oestrogen-dependant lung cancer development. There is considerable evidence suggesting that the level of oestrogen in lung tumours is elevated. Therefore, by using reverse transcription, real-time PCR and Western Blot analysis, we evaluated the CYP1B1 status in tissues from 76 patients diagnosed with non-small cell lung cancer (NSCLC) to confirm whether potential overexpression of CYP1B1 may impact lung cancerogenesis induced by oestrogens. We found significantly lower levels of CYP1B1 transcripts ($p = 0.00001$) and proteins ($p = 0.000085$) in lung tumour material compared to corresponding, histopathologically unchanged tissues. We also analysed the association of CYP1B1 expression with gender, age and clinicopathological data of NSCLC patients. We observed lower amounts of CYP1B1 occurring in the middle stages of LC, regardless of gender, age or histological type of lung cancer.

1. Introduction

Lung cancer (LC) is the most frequently diagnosed type of tumour, and despite current improvements in diagnostics and therapies, it is the most common cause of cancer-related death among men and women [1]. Because it develops slowly and does not display any specific symptoms, the main reason for such a low survival rate is late diagnosis, resulting in the detection of disease at advanced stages, leaving very limited treatment options [2–4].

Clinical classification of LC distinguishes its two main histopathological types: non-small cell lung cancer (NSCLC), which occurs in 80% of lung cancers, and small cell lung cancer (SCLC), which occurs in 20% of cases. NSCLC can be divided into several subtypes, with adenocarcinoma (ADC), squamous cell carcinoma (SSC) and large cell carcinoma being the most common forms [4].

LC is one of the few cancers caused mainly by unhealthy lifestyle. It is well known that many people afflicted by this type of cancer are former or current smokers [2,5]. The association between LC and long-term exposure to cigarette smoke or other inhalable xenobiotics, including organic solvent vapours, paints and asbestos is well-documented and remains indisputable [4–6]. However, it is important to emphasize that LC is a very complex disease and can be related to many different factors such as genetic (*EGFR* mutations [3,7,8]) and chromosomal (*EML-ALK* oncogene fusion [8]) anomalies or environmental pollution [4,5]. Recent reports have also provided a large amount of evidence that the occurrence and progression of LC can be affected by oestrogens [7,9–12]. By binding to the oestrogen receptor (ER), oestrogen can initiate a cellular signal that is followed by transcription of the oestrogen-dependant genes or activation of various cytoplasmic kinases (depending on whether a genomic or non-genomic response is triggered). This process subsequently leads to intensified cell proliferation [13–16].

Many investigations have showed significant changes in the tumoural expression of enzymes involved in oestrogen *in situ* synthesis (as compared to histopathologically unchanged material), including increased levels of 17-beta-hydroxysteroid dehydrogenase type 1 (HSD17β1) and aromatase (CYP19A1). These disturbances in expression of the aforementioned proteins may lead to changes in intracellular levels of 17β-oestradiol (E_2) and thus could be the reason for enhanced cancerogenesis [10,12,17,18]. Apart from being involved in the receptor-dependant cellular response, oestrogen, if present in cytoplasm, can be transformed to genotoxic metabolites through cytochrome P450

* Corresponding author.
E-mail address: slowikowski.bartek@gmail.com (B.K. Słowikowski).

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1B1 (CYP1B1) activity [19]. This enzyme is responsible for hydroxylation of oestrogens at their 2- and 4-positions, which is followed by the formation of genotoxic catechol derivatives such as 4-hydroxyestradiol (4-OHE_2). Additionally, once produced, the endogenous oestrogen metabolites can be oxidized by any enzyme with oxidative activity [20–22]. Afterwards, this process promotes the generation of reactive electrophilic oestrogen quinone-derivatives [23,24]. All of these compounds may harm cells in several ways. First, the enzymatic transformation of the quinones can result in the formation of free radicals, particularly hydroxyl radicals, which are known as the most harmful oxidizing factor [21,23–25]. The unwelcome presence of these particles can cause several types of DNA damage, i.e., single strand breaks, chromosomal anomalies and the creation of 8-Oxo-2′-deoxyguanosine (8-oxo-dG), the most common DNA damage caused by oxidation [21,23,25]. Second, the catechol and quinone derivatives of oestrogens are capable of binding to the ER, which is followed by their translocation to the nucleus. This process results in depurination of DNA, its mutagenesis and formation of any other damage related to emission of the free radicals [15,26,27].

It should be emphasized that CYP1B1 is not only involved in oestrogen transformation but is also responsible for activation of procarcinogens present in tobacco smoke to their cancerogenic form. Moreover, CYP1B1 expression is induced by long-lasting tobacco combustion [28]. As reported, it is presumed that the presence of oestrogen may synergize with the mutagenic properties of tobacco smoke, through induction of CYP1B1 expression which may subsequently lead to intensified carcinogenesis.

Verma et al. and Drzewiecka et al. each independently reported that tumour tissue, compared to histopahologically normal tissue, is characterized by the elevated expression of enzymes responsible for a possible intracellular increase in E_2 [10,12,18]. In light of these data, we decided to analyse the status of CYP1B1 in LC tissue and in corresponding healthy tissue to confirm if potential overexpression of CYP1B1 can affect cancerogenesis induced by oestrogens.

2. Material and methods

2.1. Patient material

Lung cancer tissue was obtained from 76 patients who had been diagnosed with NSCLC. The material was collected during surgical intervention at the Department of Thoracic Surgery, Poznan University of Medical Sciences, Poland. No chemo- nor radiotherapy was given to any patient before the surgical procedure. The non-pathogenic material was obtained from the same patients from macroscopically unchanged areas at least 10–20 cm away from cancer foci. The histopathological recognition was performed by an experienced pathologist who confirmed the type and stage of the neoplastic lesion by macroscopic and microscopic examination. Histopathological evaluation was based on The TNM Classification of Malignant Tumours (TNM; T – Primary Tumours; N – Regional Lymph Nodes; M – Distant Metastasis) specified in the 7th Edition of the AJCC Cancer Staging Manual of The American Joint Committee on Cancer [29,30] and the cancer stage was determined according to the aforementioned guidelines. After surgical removal, the tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until they were used for protein/RNA isolation purposes. A total of 87.8% of patients declared being current or former smokers with long smoking histories (Table 5). All patients provided written and oral consent to participate in this research under a protocol which was approved by the Local Ethics Review Board of Poznan University of Medical Sciences.

2.2. RNA isolation, reverse transcription and real-time quantitative polymerase chain reaction (qPCR) analysis

Total cellular RNA from previously homogenized NSCLC tissue samples was isolated according to the Chomczyński-Sacchi protocol [31]. The concentration and purity of RNA were established spectrophotometrically (NanoDrop™ One Microvolume UV–vis Spectrophotometer, Thermo Fisher Scientific, Waltham, USA). After the isolation procedure, agarose gel (1.,5%) electrophoresis was performed to determine the integrity of the RNA. The isolated RNA was stored at −80 °C until further analysis.

Complementary DNA (cDNA) was synthesized by using SuperScript IV Reverse Transcriptase according to manufacturer's protocol (Thermo Fisher Scientific). Quantitative real-time PCR was carried out in the Light Cycler® 480 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR green I as the detection dye (LightCycler® 480 SYBR Green I Master, Roche Diagnostics GmbH). The reaction efficiencies were calculated by generating standard curves from serial dilution of cDNA template mix from all of the patients' samples. The negative, no-template, non-transcribed RNA and DNA controls were included. To calibrate the reaction, we used 1 µl of mixed cDNA's from all the tissue samples. The quantity of *CYP1B1* transcript in each reaction was standardized with the geometric mean of *porphobilinogen deaminase* (*PBGD*), *human mitochondrial ribosomal protein L19* (*hMRPL19*) and *RNA polymerase II subunit A* (*POLR2A*) cDNA levels. To confirm the specificity of the qPCR product, melting curve analysis and agarose gel electrophoresis were performed. All experiments were repeated three times, and *CYP1B1* transcript level was expressed as a decimal logarithm of multiplicity of cDNA concentrations in the calibrator.

2.3. Protein isolation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting analysis

For protein isolation, tissue specimens were homogenized in liquid nitrogen and then treated with RIPA lysis buffer (Sigma-Aldrich, St. Louis, USA) supplemented with protease inhibitor (Roche Diagnostics GmbH,). Next, proteins were resuspended in sample loading buffer, incubated at 98 °C and separated on 8% Tris-Glycine gels, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto nitrocellulose membranes. Nitrocellulose membranes were then blocked with 5 % non-fat dry milk in 1 × concentrated Tris-buffered saline/Tween 20 for 1.,5 h at room temperature. After blocking, membranes were incubated overnight in 4 °C with rabbit-polyclonal anti-CYP1B1 (PA523139, Thermo Fisher Scientific) antibody at the dilution 1:1000. The membranes were then washed in the 1 × concentrated Tris-buffered saline/Tween 20 and placed in a solution (1:5000) of secondary anti-rabbit HRP-conjugated antibody. To limit the unspecific signal derived from globulins present in blood plasma, we used, secondary antibodies (F(ab')2-Goat anti-Rabbit IgG (H + L) Secondary Antibody, HRP conjugate, A24537, Thermo Fisher Scientific,). The immunochemiluminescent signal was revealed by using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific,) and the Biospectrum® Imaging System 500, UVP Ltd. (UVP Ltd., Upland, USA). The experiment included MCF-7 protein lysate as a positive control. Afterwards, the membranes were restripped and incubated overnight with rabbit anti-GAPDH antibody (Santa Cruz Biotechnology, USA) at the dilution of 1:3300 followed by reaction with secondary anti-rabbit antibody (1:5000) (Santa Cruz Biotechnology, USA). The amounts of analysed proteins were presented as the decimal logarithms of CYP1B1-to-GAPDH band optical density ratio.

2.4. Statistical analysis

The normality of the observed patient data distribution was assessed by the Shapiro-Wilk test. The Mann-Whitney *U* test was used to compare the mean values of CYP1B1 transcript and protein level and consider statistically significant differences between lung tumour tissue and histopathologically unchanged material. The Multivariate regression of the Kruskal–Wallis (three or more variables) or Mann-Whitney U