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Genetic and microscopic assessment of the human chemotherapy-exposed placenta reveals possible pathways contributive to fetal growth restriction

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ABSTRACT

Introduction: Fetal growth restriction (FGR) carries an increased risk of perinatal mortality and morbidity. A major cause of FGR is placental insufficiency. After in utero chemotherapy-exposure, an increased incidence of FGR has been reported. In a prospective cohort study we aimed to explore which pathways may contribute to chemotherapy-associated FGR.
Methods: Placental biopsies were collected from 25 cancer patients treated with chemotherapy during pregnancy, and from 66 control patients. Differentially expressed pathways between chemotherapy-exposed patients and controls were examined by whole transcriptome shotgun sequencing (WTSS) and Ingenuity Pathway Analysis (IPA). Immunohistochemical studies for 8-OHdG and eNOS (oxidative DNA damage), proliferation (PCNA) and apoptosis (Cleaved Caspase 3) were performed. The expression level of eNOS, PCNA and IGFBP6 was verified by real-time quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR).
Results: Most differential expressed genes between chemotherapy-exposed patients and controls were related to growth, developmental processes, and radical scavenging networks. The duration of chemotherapy exposure had an additional impact on the expression of genes related to the superoxide radicals degeneration network. Immunohistochemical analyses showed a significantly increased expression of 8-OHdG ($P = 0.003$) and a decreased expression of eNOS ($P=0.015$) in the syncytiotrophoblast of the placenta of cancer patients. A decreased expression of PCNA was detected by immunohistochemistry as RT-qPCR (*NS*).
Conclusion: Chemotherapy exposure during pregnancy results in an increase of oxidative DNA damage and might impact the placental cellular growth and development, resulting in an increased incidence of FGR in this specific population. Further large prospective cohort studies and longitudinal statistical analyses are needed.

1. Introduction

Fetal growth restriction (FGR) can be caused by maternal, placental and fetal factors, or by an imbalance in the complex

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interaction between these three compartments [1]. There is evidence to suggest that impaired vasculogenesis is the most striking pathology identified in obstetrical complications and more specifically in FGR [2]. In up to 90% of all growth-restricted infants the underlying cause is placental disturbed angiogenesis and villous formation [3]. Disorders in the very precise elaboration of the uteroplacental compartment may result in an impaired trophoblast invasion and inadequate perfusion, which are known as underlying mechanisms leading to preeclampsia and FGR [4]. Correct diagnosis of FGR is important since FGR carries an increased risk of perinatal morbidity and mortality. Preterm birth, neonatal hypothermia, hypoglycemia, morbidities and even perinatal mortality can occur in the acute setting, while more cardiovascular and metabolic diseases are seen in the long-term follow-up of these children [5–7].

Studies on the outcome of children after prenatal exposure to chemotherapy show an increased risk of FGR [8–12], up to 21%. The high incidence of FGR in pregnant cancer patients may have multiple causes: diminished caloric intake, anemia, increased incidence of thrombosis, toxic (treatment) exposure and negative impact on the uteroplacental blood flow, relative older maternal age, high maternal stress levels, and/or chronic disease/inflammatory response. Animal experiments have shown that tumor growth in pregnant rats has deleterious effects on placenta and fetus [13,14]. Studies reported impaired fetal growth, changes in placental weight and protein content as well as increased hemorrhage and edema with high fetal resorption [13]. It is hypothesized that through competition for nutrients the rapid tumor growth damages the placental development and fetal growth [15]. Substances synthesized by the tumor may cause oxidative stress reactions resulting in an increased ratio of apoptotic trophoblast [14]. In 2009 Abellar R.G. et al. described the pathologic findings in 13 placentas exposed to chemotherapy and observed histologic findings suggestive of placental underdevelopment when chemotherapy was administered during 2nd and 3rd trimesters of pregnancy [16]. However, they also indicated that other detrimental factors (malnutrition, stress, immune suppression) might have had an additional impact.

In this study, we investigated the placental physiology and pathology in a small prospective exploratory cohort study of patients diagnosed and treated for cancer during pregnancy to identify possible mechanisms of chemotherapy-associated FGR. With the use of whole transcriptome shotgun sequencing (WTSS) we explored the presence of important activated or depressed pathways in the placental tissue after chemotherapy-exposure. In addition immunohistochemical and expression analyses by Reverse Transcription Polymerase Chain Reaction (RT-qPCR) were performed to explore some factors related to the differential expressed pathways (oxidative DNA damage, apoptosis, proliferation).

2. Methods

2.1. Patients and data collection

Cancer patients and controls were prospectively recruited during pregnancy. Between January 2014 and September 2016, cancer patients, all treated with chemotherapy during pregnancy, were recruited from Belgium (n = 19), the Netherlands (n = 3), the Czech Republic (n = 2), and Luxembourg (n = 1). Controls (n = 66) were recruited from the University Hospital Leuven and the University Hospital of Louvain-la-Neuve. All newly registered cancer patients were entered in the study after obtaining a written informed consent; allocation to the FGR or no FGR group took place after delivery. Birth weight percentiles were calculated considering the gestational age at birth, birth weight, sex, ethnicity, parity, and maternal length and weight (www.gestation.net, v6.7.5.7(NL),2014). A percentile <10 was considered as FGR. FGR controls were recruited based on sonographic Estimated Fetal Weight (EFW) below the 10th percentile, measured after 30 weeks of pregnancy. If after delivery the birth weight percentile turned out to be above 10, the patient was excluded from the FGR control group. Normal weight (NW) controls were recruited at admission to the delivery room. Exclusion criteria for all controls were: maternal medical disorders (Crohn's Disease, colitis, congenital heart disorders, auto-immune disease) and presence of Doppler abnormalities mostly due to early (<30 weeks GA) and severe preeclampsia. The cancer (1) and control (2) patients were subdivided in FGR (A) and NW (B). The study was approved by the Ethical Committee of University Hospital Leuven (Belgian number B322201421061/S56168). Detailed general, obstetric and oncological information was available from the online registry database (www.incipregistration.be) and from patient files. Recorded patient characteristics included maternal age, ethnicity (Caucasian, North-African, African, Asian or Latin-American), maternal body mass index (BMI), cigarette smoking during pregnancy (yes/no), obstetrical complications (including hypertensive disorders, diabetes, preterm labor, maternal infection, or cholestasis (yes/no)), parity (nulli- or multiparous), gender of the neonate, gestational age (GA) at birth (days), birth weight and percentile.

The placentas were weighted immediately after delivery. Placental samples from each patient were taken from 4 different cotyledons (4 quadrants) at the maternal side of the placenta. Each sample was divided in 2 parts and rinsed in phosphate buffered saline (PBS) before storage in RNAse buffer (RNA stabilization reagent, Qiagen, Hilden, Germany) and fixation in 4% buffered formaldehyde respectively. Samples in RNAse buffer were stored for maximally 4 weeks at 4 °C until analysis. while fixed samples were processed for microscopic examination. All laboratory analyses were performed after validation of the methods and, where possible, samples were tested simultaneously to minimize inter-essay variability.

2.2. RNA extraction from placental tissue, whole transcriptome shotgun sequencing (WTSS) and pathway analysis

Total RNA was isolated using Tripure Isolation Reagent (Sigma-Alldrich, Bornem, Belgium). The quantity of the extracted RNA was photometrically tested (NanoDrop - Isogen Life Science, Temse, Belgium). The quality of the extracted RNA was evaluated by an RNA integrity assay system (Experion RNA StdSens Analysing kit - Bio-Rad, Nazareth Eke, Belgium: good quality RNA samples included an RNA integrity number > 7 for all samples). One μg of total RNA was used as input material for sequencing library preparation which was performed with the Illumina TruSeq Stranded mRNA Sample Preparation Kit according to the manufacturers protocol. RNA was denaturated at 65 °C in a thermocycler and cooled down to 4 °C. Samples were indexed to allow for multiplexing. Sequencing libraries were quantified using the Qubit fluorometer (Thermo Fisher Scientific, Massachusetts, USA). Library quality and size range was assessed using the Bioanalyser with the DNA 1000 kit (Agilent Technologies, California, USA) according to the manufacturer's recommendations. Each library was diluted to a final concentration of 2 nM and sequenced on Illumina HiSeq2500 according to the manufacturer's recommendations generating 50 bp single-end reads. Adapters from raw reads were filtered with software ea-utils v1.2.2 [17]. Raw reads were aligned to the reference human genome hg19 with Tophat v2.0.13 [18]. Quantification of reads per gene and downstream inference analyses with DeSeq2 [19] were performed using the software Array Studio V10.0 (Qiagen, Hilden, Germany). Fold change of gene expression among different groups, and the corresponding corrected p-value or False