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Research paper

Unravelling the link between embryogenesis and cancer metastasis

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ABSTRACT

Purpose: Cancer as opposed to embryonic development is characterized by dysregulated, uncontrolled and clonal growth of cells. Inspite of that they share certain commonality in gene expression patterns and a number of cellular & molecular features. Consequently, in the present study we aimed to evaluate the role of a definite set of genes in fetal liver, primary liver cancers and metastatic liver tissue.

Methods: The relative expression of fourteen candidate genes obtained by data mining and manual curation of published data (*CXCL12*, *CXCR4*, *CK7*, *CDH1*, *CTNNB1*, *CLDN4*, *VEGFA*, *HIF1A*, *MMP9*, *p53*, *OPN*, *CDKN2A*, *TGFBR2*, *MUC16*, *β-actin*) were performed on 62 tissues (32 liver metastasis tissues and 30 primary Liver cancer tissues), Fetal liver tissues (below and above 20 weeks of gestation) and 2 sets of control samples by real-time quantitative reverse transcription PCR (qRT-PCR).

Results: Results showed significant down-regulation of *MMP9* and *TP53* in Fetal liver above 20 weeks of gestation whereas it was up-regulated in fetal liver below 20 weeks of gestation, primary liver cancers and liver metastasis. Contradictory to that *OPN* and *CDKN2A* were significantly up-regulated in primary liver cancer, liver metastasis; down-regulated in fetal liver above 20 weeks of gestation but were not expressed during early embryo development (below 20 weeks of gestation). Moreover, *MMP9* and *TP53* demonstrated a strong correlation with *MUC16* whereas *CDKN2A* and *OPN* showed correlation with *CXCL12/CXCR4* signifying that *MUC16*, *CXCL12/CXCR4* might be involved in the complex process of cancer metastasis.

Conclusion: *MMP9*, *OPN*, *TP53* and *CDKN2A* were the identified markers that were expressed in a similar pattern in early embryonic development and cancer development & invasion suggesting that these genes are activated during embryogenesis and might be re-expressed in cancer metastasis. Moreover, these genes govern a pathway that might be activated during cancer metastasis. Thus, targeting these molecules may provide better treatment for metastatic liver cancers.

1. Introduction

Mammalian embryo development encompasses an intricate series of events to form complex higher-order organisms (Soundararajan et al., 2015). Much like the multi-step process of tumor progression to metastasis the constituent cells of the embryo called the trophoblastic cells, share characteristics with the malignant cells such as cell proliferation, cell differentiation, lack of cell-contact inhibition, migration, invasion as well as their capacity to escape effectors of the immune system particularly in the first trimester of pregnancy. These properties have led to the definition of trophoblasts as 'pseudo-malignant' tissue or as 'physiological metastasis' (Genbacev et al., 1997; Mullen, 1998; Ferretti et al., 2007). Moreover, many key molecules commonly expressed by the early embryos and the metastatic cells are known to gauge genes and proteins that participate in molecular circuits implicated to achieve their proliferative, migratory and invasive properties (Calvoa and Drabkin, 2000; Ferretti et al., 2007).

In the past decade development of a number of gene expression signatures related to embryo development, cell migration, stemness or EMT are investigated for patient outcome predictions. These examinations comprise a wide variety of cell types such as – cells of the embryonic origin, cell lines that exhibit stem-cell features, cells which display classic EMT, and include different methods of EMT/stemness/pluripotency induction. Moreover, genome profiling/microarray/

Abbreviations: EMT, epithelial mesenchymal transition; qRT-PCR, quantitative real time polymerase chain reaction; CXCL12, C-X-C motif chemokine ligand 12; CXCR4, C-X-C chemokine receptor type 4; CK7, cytokeratin-7; CDH1, E-cadherin; CLDN4, claudin-4; HIF-1A, hypoxia induced factor-1A; VEGFA, vascular endothelial growth factor A; MMP-9, matrix metalloproteinases 9; OPN, osteopontin; SPP1, secreted phospho protein 1; CDKN2A, cyclin dependent kinase inhibitor 2A; TGFBR2, transforming growth factor beta receptor, type II; MUC16, mucin-16; TP53, tumor protein 53; CTNNB1, beta catenin

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Table 1
Sequences and primer sets used for Real Time PCR.

Gene	Forward primers	Reverse primers
CXCL12	5′-AAGCCCGTCAGCCTGAGCTA-3′	5′-TTAGCTTCGGGTCAATGCACAC-3′
CXCR4	5′-AATAAAATCTTCCTGCCCACC-3′	5′-CTGTACTTGTCCGTCATGCTTC-3′
CK7	5′-GACATCGAGATCGCCACCTAC-3′	5′-ATTGCTGCCCATGGTTCCC-3′
CDH1	5′-GACTCGTAACGACGTTGCAC-3′	5′ -GGTCAGTATCAGCCGCTTTC-3′
CTNNB1	5′-TGGATACCTCCCAAGTCCTG-3′	5′-CAGGGAACATAGCAGCTCGT-3′
CLDN4	5′-AGATGGGTGCCTCGCTCTAC-3′	5′-CCAGGGAAGAACAAAGCAGA-3′
HIF-1α	5′–ACAGCCTCACCAAACAGAGCAG-3′	5′-CGCTTTCTCTGAGCATTCTGCAAAGC-3′
VEGFA	5′-CTTGCCTTGCTGCTCTACC-3′	5′-CACACAGGATGGCTTGAAG-3′
MMP9	5′-GAGTGGCAGGGGGAAGATGC-3′,	5′-CCTCAGGGCACTGCAGGATG-3′
p53	5′-CCGTGTTGGTTCATCCCTGTA-3′	5′-TTTTGGATTTTTAAGACAGAGTCTTTGTA-3′.
OPN	5′-ACTCGTCTCAGGC CAGTTG-3′	5′-CGTTGGACTTGGAAGG-3′
CDKN2A	5′-CCCAACGCACCGAATAGT-3′	5′-GGGGATGTCTGAGGGACCTT-3′
TGFβR2	5′-GTAGCTCTGATGAGTGCAATGAC-3′	5′-CAGATATGGCAACTCCCAGTG-3′
MUC16	5′-CTGAGACCCCAACATCCTTG-3′	5′-GGTCACTAGCGTTCCATCAG-3′
β-Actin	5′-TGACGTGGACATCCGCAAAG-3′	5′-CTGGAAGGTGGACAGCGAGG-3′

retrospective qRT-PCR analyses of a defined set of cancer-related genes from patient samples are also performed. All these resources have their own advantage, but either they have not been tested for metastasis predictions, or are unfortunately quite limited in their potential to accurately predict the metastatic relapse/spread of tumors (Monk and Holding, 2001; Kim and Orkin, 2011; Karlsson et al., 2014).

In our previous study involving meta-analysis of the microarray data sets a panel of 13 genes which were showing differential gene expression was predicted (Shah et al., 2017). We hypothesized that metastatic cells might be the result of reactivation of the repressed embryonic genes. With the results above and the literature curation, 14 putative genes (*CXCL12*, *CXCR4*, *CK7*, *E-cadherin*, *β-catenin*, *Claudin-4*, *HIF-1A*, *VEGFA*, *MMP-9*, *p53*, *OPN*, *CDKN2A*, *TGFBR2*, *MUC16*) are selected to validate in Fetal liver (before and after 20 weeks), primary liver cancers and liver metastasis tissues. Furthermore, we performed statistical analysis and correlation amongst the various genes and found that a differential gene expression pattern was observed amongst different sample cohorts.

Our results led to the identification of various signalling pathways and molecules that could provide novel targets for diagnosis and treatment of metastatic diseases that mimics the embryogenesis process thus enabling the translation of basic research discoveries into clinical applications.

2. Materials and methodology

2.1. Study population

A total number of 62 liver tissue samples were analyzed in the present study. Fresh tissue samples were collected from the radiology clinic during routine USG guided FNAC liver biopsy. More precisely, the study included 30 tissue samples from primary liver cancers (3 cholangiocarcinomas & 27 Hepatocellular carcinomas) and 32 tissue samples from patients with liver metastasis between January 2016 and October 2016. The presence of primary liver tumor or metastasis was verified histo-pathologically and only those samples that were conceded the criteria were included in the study. Moreover, following sampling the tissues were sectioned into two mirror image specimens. One of the sections was always evaluated by the pathology unit, in order to confirm the presence or absence of malignancy in the tumor specimen. The other tissue specimens were immediately frozen in liquid nitrogen after the biopsy and stored at − 80 °C until further use. None of the patients received either chemotherapy or radiotherapy. RNA extracted from Fetal Liver - below 20 weeks (Agilent Technologies, USA) and above 20 weeks (Clontech, Takara Bio Company, USA) was commercially obtained for this study. Also, RNA extracted from normal liver (Agilent Technologies, USA & Clontech, Takara Bio Company, USA) was used as control for the study.

The study was performed according to the ethical standards of the 1975 Declaration of Helsinki, as revised in 2008. Informed consent was obtained from individual cases prior to sampling as the study was approved by the Institutional Review board and Ethics committee at the Gujarat Cancer & Research Institute.

2.2. Extraction of total RNA

Total RNA was extracted from 25 mg tumor tissue using RNeasy tissue kit (Qiagen 74106) based on a silica gel membrane technology by selective binding, stepwise washing, and elution of RNA following manufacturer's instructions. A digestion step on the spin column was performed using RNase-free DNase set (Qiagen 79254). The concentration of the isolated RNA was quantified with Qubit 3.0 Fluorometer (Invitrogen by Life Technologies, CA, USA). RNA integrity was further checked on 1% FA gels on Mini-Sub-Cell GT (BioRad) and was visualized on Gel Documentation System (BioRad; Gel Doc XR +). The extracted RNA was then stored at − 80 °C until further analysis. Thereafter, total 1 μg RNA was reverse transcribed to cDNA using the cDNA archive kit (Applied Biosystems – ABI; Cat no: 4368813) in 50 μl reaction volume using manufacturer's instructions and the resulting first strand cDNA was used as a template for Real Time PCR analysis.

2.3. Real time PCR (qRT-PCR)

SYBR green-based real-time quantitative polymerase chain reaction assays were used for the gene expression analysis using gene specific primers for *CXCL12*, *CXCR4*, *CK7*, *CDH1*, *CTNNB1*, *CLDN4*, *HIF-1A*, *VEGFA*, *MMP-9*, *p53*, *OPN*, *CDKN2A*, *TGFBR2*, *MUC16* and *β-actin* as housekeeping gene as shown in Table 1. The reaction mixtures (20 μl) consisted of 10 μl Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, USA), 0.5 μl (200 nM) each of the forward primer & reverse primer, and 2 μl cDNA. β-actin gene expression was measured as endogenous control and all the primers were custom ordered using the following sequences [Table 1]. Amplification was performed using AriaMx Real-time PCR System (Agilent Technologies, USA) at the following cycling conditions: 1 cycle of 3 min at 95 °C for the initial denaturation step and 40 cycles of 5 s at 95 °C for the denaturation step, 20 s at 60 °C for the annealing and extension step. Melting curve analysis and agarose gel electrophoresis were performed following the amplification in order to distinguish the accumulation of specific reaction products from non-specific products or primer dimmers.

The generation of a specific PCR product was tested using the ΔΔCT method and *β-actin* was used as the endogenous control. All experiments were performed in triplicate independently and average C_T value