QUANTITATIVE ANALYSIS OF SIX GENE PRODUCTS AS CANDIDATE MARKERS OF EARLY PLACENTAL VILLI DEVELOPMENT IN THE HUMAN

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Abstract: Early placental development is critical for successful pregnancy. Recently, we have reported that ~70 genes were differentially expressed in human placental villi between 6- and 8-weeks of gestation in cDNA-based expression arrays for ~400 PCR products, of which six specific gene products (COL4A4, CXCR4, ERBB2, HDAC1, HPRT1, and TNFRSF1A) appeared intriguing. In the present study we have examined expressions of these six candidate genes in placental villi obtained from 6-weeks, 7-weeks and 8-weeks (n = 6 for each group) human placental samples using quantitative real time RT-PCR. We observed that there was considerable concordance (>95% confidence) in pair-wise analysis of transcript profiles between the two methods, however, absolute quantitative values as measured by quantitative RT-PCR differed from those obtained from cDNA-based array analysis for 2 gene products (CXCR4 and ERBB2) out of 6 genes. No significant change was observed in the steady state expression of COL4A4 and HPRT1 during the time period examined. However, there was significant decrease in CXCR4 for 7-weeks (P< 0.01) and 8-weeks (P<0.05) samples, and significant (P<0.05) increase was seen for ERBB2 in 7-weeks and 8-weeks as compared to 6-weeks samples with no change between 7-weeks and 8-weeks samples. Moreover, significant (P< 0.05) increase for HDAC1 and decrease for TNFRSF1A was observed in 8-weeks samples as compared to 6-weeks samples with no change observed between 6-weeks and 7-weeks samples. We infer that it is essential that cDNA array-based data are verified in terms of quantitative estimates preferably by quantitative PCR before their use for any exploratory purpose. Taking together our previous array based data and the present study we conclude that a categorical balance exists between the expression of ERBB2 and HDAC1 genes affecting cell proliferation and differentiation in one hand, and CXCR4 and TNFRSF1A affecting chemotaxis, inflammatory response and apoptosis on the other hand. The expression of these genes appear important for the early development of human placental villi.

Key words: early placenta villi gene expression transcriptomics

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INTRODUCTION

Early placental development is critical for successful pregnancy in the human. Developmental inadequacy in placenta at this stage is associated with more than 50% incidence of pregnancy loss during the first trimester, and also with increased risk of developing several types of complications during later stages of pregnancy like preeclampsia, intrauterine growth retardation and spontaneous abortion (1, 2). We have earlier reported that human placenta villi during 6- to 8-weeks of gestation display a complex pattern of homeodynamics involving proliferation, development associated syncytialization and apoptosis (3). Recently, we have examined the transcript expression patterns of human placental villi of normal pregnancies during 6- to 8-weeks of gestation using custom-tailored cDNA-based expression arrays for ~400 gene products assumed to be important during the early pregnancy, and observed that a large number (70) of gene products that are known to be involved in cell growth and proliferation, apoptosis, angiogenesis, immune and inflammatory responses, extracellular matrix remodeling and multicellular organismal development showed overt expression in placental villi between 6 and 8 weeks (4). Of these gene products, six specific gene products as shown in Table I appeared intriguing. In the present study we have examined expressions of these six candidate genes (COL4A4, CXCR4, ERBB2, HDAC1, HPRT1, and TNFRSF1A) using quantitative real time RTPCR to examine whether these gene products were indeed over-represented in the early stage placental villi. We report here for the first time that categorical regulation of genes responsible

<table>
<thead>
<tr>
<th>Symbol of candidate gene</th>
<th>Description of gene products</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL4A4 (D17391)</td>
<td>Collagen alpha-4(IV) chain is a protein encoded by the COL4A4 in the human. It constitutes one of the six subunits of type IV collagen that forms the major structural component of basement membranes (5).</td>
</tr>
<tr>
<td>CXCR4 (D10924)</td>
<td>CXC chemokine receptor type 4, also called fusin, is a protein encoded by the CXCR4 gene in the human. It is an alpha-chemokine receptor specific for stromal-derived-factor 1 (SDF-1). This chemokine receptor is involved in chemotaxis, inflammation, embryo adhesion to endometrium, and mobilization of stem cells (7).</td>
</tr>
<tr>
<td>ERBB2 (NM_001005862)</td>
<td>The gene encodes for a member of the ErbB protein family, and more commonly known as the epidermal growth factor receptor family. It is a cell membrane surface bound receptor tyrosine kinase and is normally involved in the signal transduction pathways leading to cell growth and differentiation. Its expression is regulated by estrogen receptor activity. It reportedly interacts with a large number of intracellular regulatory factors (9).</td>
</tr>
<tr>
<td>HDAC1 (NM_004964)</td>
<td>Histone deacetylase 1 is an enzyme encoded by the HDAC1 gene in the human and found primarily in the nucleus. It is a component of multisubunit complex of histone deacetylase complex and catalyzes histone acetylation and deacetylation and thereby regulates eukaryotic gene expression (10).</td>
</tr>
<tr>
<td>HPRT1 (NM_000194)</td>
<td>The product of housekeeping HPRT1 gene present in chromosome X in the human is hypoxanthine-guanine phosphoribosyl transferase which is a cytosolic enzyme involved in purine metabolism primarily in the salvage pathway. Mutations in HPRT1 gene results in a rare inherited disorder, Lesch-Nyhan syndrome (11).</td>
</tr>
<tr>
<td>TNFRSF1A (NM_001065)</td>
<td>The gene encodes for tumor necrosis factor superfamily member 1A protein which is a major receptor for tumor necrosis factor alpha. This receptor can activate the transcription factor NF-kB, mediate apoptosis, and function as a regulator of inflammation, as well as, in the receptor mediated signal transduction (12).</td>
</tr>
</tbody>
</table>

1For array results see reference 4. 2according to HUGO Gene Nomenclature Committee (HGNC). 3direct submission by Sugimoto et al. (6). 4direct submission by Nomura et al. (8).
for cell proliferation and differentiation \((ERBB2\) and \(HDAC1\)) in one hand, and that of genes responsible for inflammatory responses and apoptosis \((CXC4\) and \(TNFRSF1A\)) on the other hand is required for normal development of placental villi during 6-8 weeks of gestation. Moreover, sustained expression of genes for housekeeping activities, for example \(COL4A4\) and \(HPRT1\), are integral to the early stage developmental program of human placental villi.

**MATERIALS AND METHODS**

**Tissue samples**

The details of collection and isolation villous samples from first trimester human placental samples have been described previously \((3, 4)\). In brief, placental samples were obtained from twenty four healthy proven fertile women within age group of 23-30 years undergoing elective surgical termination of pregnancy during 6- to 8-weeks of gestation without any prior medication at the All India Institute of Medical Sciences, New Delhi. The gestational age was estimated from menstrual history, physical examination and ultrasonographic evaluation. All women had voluntarily donated their samples after understanding the purpose of the proposed study and gave their written consent. The Ethics Committee of the All India Institute of Medical Sciences had approved the research study. Placental villi were isolated from freshly collected placentas and used for RNA extraction as described previously \((4)\). Table II shows the details of the samples.

**RNA extraction**

The methodological details of RNA extraction have been given elsewhere \((4, 13)\). Briefly, total RNA was extracted using Trizol \((\text{Agilent Technologies Singapore Pvt. Ltd., Shung Avenue, Singapore})\) and cleaned up with DNAse I \((\text{Sigma Chemical Co., St. Louis, Missouri, USA})\) and subjected to re-extraction when it was necessary. The yield and purity of the extracted RNA was checked using standard protocols of spectrophotometric absorbance at 260 nm, ratio between 260 nm and 280 nm \((\geq 1.8)\) and 1% agarose gel electrophoresis \((14)\). Furthermore, RIN score of individual sample was determined using Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip kit and Agilent 2100 Expert Software \((\text{Agilent Technologies, Inc., Santa Clara, CA, USA})\) \((15)\). Four samples that could not yield either sufficient amount of RNA or acceptable RIN score \((\geq 8.0)\) were discarded \((\text{Table 2})\). All accessory chemicals were purchased from Sigma Chemical Co. \((\text{St. Louis, Missouri, USA})\) and Invitrogen \((\text{Agilent Technologies Singapore Pvt. Ltd., Shung Avenue, Singapore})\).

**Quantitative reverse transcription polymerase chain reaction (Q-RTPCR)**

The relative expression of six selected genes inclusive of actin-beta as the endogenous control in all samples were

<table>
<thead>
<tr>
<th>Group</th>
<th>Description (weeks of gestation)</th>
<th>Samples (number)</th>
<th>Collected</th>
<th>Extracted(^1)</th>
<th>Selected(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>6</td>
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<tr>
<td>3</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td></td>
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</tbody>
</table>

\(^1\)Shows numbers of samples that yielded sufficient villi in the respective groups.

\(^2\)Based on RNA quality assessment from electrophoresis, yield and RIN score \((\geq 8.0)\) as described in Materials and Methods.

\(ERBB2\) and \(HDAC1\) for cell proliferation and differentiation in placental villi. \(CXC4\) and \(TNFRSF1A\) for inflammatory responses and apoptosis.
performed using real time RTPCR technology on a BioRad platform (iCycler iQTM Real time PCR detection system, BioRad, Hercules, CA, USA). Actin-beta was selected as an endogenous control based on its observed expression consistency in arrays as described previously (4). Primers and labeled probes were designed on Beacon Designer software (Labware Scientific Inc., Milpitas, CA, USA) and obtained from Qiagen (Cologne, Germany). Details of primers and probes for target genes are given in Table III. An optimized kit (Quantitect multiplex PCR kit, Qiagen, Cologne, Germany) was used according to the protocol given by the manufacturer. Briefly, cDNA was synthesized with Superscript II from same total RNA (5 μg) samples that were used for the array experiments and levels of selected transcripts were estimated by TaqMan analysis for each cDNA sample. Cycle threshold (Ct) values were obtained and ΔCt values between experimental Ct and normalization Ct were determined and relative expression ratios between groups were determined using a method described previously (16). Absolute values were derived in arbitrary units relative to the level of the same gene in standard total RNA extracted from placental villi and standard RNA supplied by Agilent Technologies (Santa Clara, CA, USA) (17).

Statistical analysis

Statistical analyses of Q-RTPCR data from all groups were done using non-parametric equivalent of analysis of variance (Kruskal-Wallis) test followed by multiple comparison test based on rank sums Wilcoxon test (18). The individual time profiles for transcript species were obtained from individual data sets by polynomial adjustment for best fit curve (19). Lin’s concordance correlation coefficients (r⊙) between values of transcript abundance obtained from arrays and quantitative RT-PCR for six selected genes were estimated to assess the reproducibility assurance of signal estimation from arrays (20). The statistical analysis was performed using SPSS software version 13 (SPSS, Inc., Chicago, Illinois, USA).

### Table III: Primers and probes used in the quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>GeneBank ID for mRNA accession</th>
<th>Primer [as/s, Tm]</th>
<th>Probes [Tm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>(XM00351)</td>
<td>CCATCTCTTGCTGAGAAGTCCAG [as, 59.3]</td>
<td>CGGCTACAGCTTACCACACACGGC [68.9]</td>
</tr>
<tr>
<td>COL4A4</td>
<td>(D17391)</td>
<td>CTGAGTTGAGCCCCATGAGAC [as, 58.8]</td>
<td>CAGTCCCATATGACACACACACCC [67.4]</td>
</tr>
<tr>
<td>CXCR4</td>
<td>(D10924)</td>
<td>TGTCCTCCTCTTCTCTCTT [as, 59.7]</td>
<td>AACCTGCCCCGACACGCTTCAC [68.4]</td>
</tr>
<tr>
<td>ERBB2</td>
<td>(NM_001005862)</td>
<td>GAGGAGGAGCCCGAGGAGATC [as, 59.4]</td>
<td>AGCAGACCCCGACGACCAAC [68.2]</td>
</tr>
<tr>
<td>HDAC1</td>
<td>(NM_004964)</td>
<td>ACCGAGGCAAAGTATTAGATT [as, 59.3]</td>
<td>CCACGCGGCTCCCAGCATACAGATA [67.6]</td>
</tr>
<tr>
<td>HPRT1</td>
<td>(NM_000194)</td>
<td>CTCAACTATCAAGACAGCAGCAAAG [as, 58.6]</td>
<td>CCACGCGGCTCCCAGCATACAGATA [67.6]</td>
</tr>
<tr>
<td>TNFRSF1A</td>
<td>(NM_001065)</td>
<td>TTTCCACCCACCCGATGTC [as, 60.2]</td>
<td>CCGGCTACAGCTTACCACACACGGC [68.9]</td>
</tr>
</tbody>
</table>

as, anti-sense; s, sense; ‘according to HGNC.
RESULTS

Table IV shows the comparisons between mean values of fold changes in inter-group analysis obtained from quantitative RTPCR and cDNA-based array and the degree of concordance in pair-wise analysis between the two methods. Thus, both methods yielded similar temporal profiles between 6-weeks and 8-weeks of gestation for relative abundance of six candidate gene products. It is however evident that, despite high degree of concordance with confidence limit more than 95% between the two methods, the absolute quantitative values as measured by quantitative RTPCR differed from the semi-quantitative values obtained from cDNA-based array analysis in gene product and tissue sample specific manners resulting in observed differences in ratio values; for example, 2-5 times differences were observed for CXCR4 for several samples, while 2-3 times differences were observed in 6-weeks to 8-weeks ratio for CXCR4, HDAC1 and TNFRSF1A.

Fig. 1 shows the polynomially interpolated quantitative profiles of selected six transcripts in human placental villi during 6-weeks to 8-weeks of gestation. Statistical analysis revealed that there was a reduction (P<0.05) in the steady state expression levels for CXCR4 (Fig. 1A) and TNFRSF1A (Fig. 1F), while significant (P<0.05) increase in the expression levels of ERBB2 (Fig. 1C) and HDAC1 (Fig. 1D) was observed in the human placental villi during 6-weeks to 8-weeks of gestation. No significant change was observed in the steady state expression profiles of COL4A4 and HPRT1 during the time period examined (Fig. 1B, E).

Multiple comparison tests revealed that the relative abundance of CXCR4 transcripts was decreased in 7-weeks (P<0.01) and 8-weeks (P<0.05) samples, while that of TNFRSF1A decreased (P<0.05) in 8-weeks samples as compared to 6-weeks samples.
with no change between 7-weeks and 8-weeks samples (Fig. 1). On the other hand, the relative abundance of ERBB2 transcripts was higher (P<0.05) in 7-weeks and 8-weeks samples, while that of HDAC1 was higher (P<0.05) in 8-weeks samples as compared to 6-weeks samples with no change between 7-weeks and 8-weeks samples (Fig. 1).

DISCUSSION

The present study highlights two major points, one relating to the technical issue of veracity of quantification of transcript levels from cDNA-based array, and the second issue relates to the early placental physiology.

Technical issue

It is generally assumed that an image scale acquired from hybridized membrane corresponds to the amount of cDNA products available for hybridization, and the amount and fidelity of cDNA products in complex probe preparation from initial RNA numbers and species are representative (21). The present study shows that, despite cDNA-based array has the merit of yielding large number of data in one go, the array technology is indeed noise-prone. The assessment of validity of estimated values for six candidate genes from cDNA-based array using TaqMan quantitative RT-PCR approach in the present study revealed that two out of six may yield discordance in the estimations. While four species of gene transcripts (COL4A4, HDAC1, HPRT1 and TNFRSF1A) showed high concordance for quantitative estimates and general profiles in 6- to 8-weeks of gestational samples, two gene transcripts (CXCR4 and ERBB2) showed only moderate concordance for quantitative estimates.

From purely an informatics point of view, a system with one-third noise predominance in the output is generally not considered as efficient (22). There are several points where cDNA-based array technique may loose relative fidelity in the representation of quantity and quality of transcripts in a primary soup. The methodology of in-vitro synthesis of complex probes using universal poly-A strategy may be intrinsically noise-prone because of sequence polymorphism, homology and palindromes (23). Furthermore,
the approach of solid phase hybridization may result in differences in signals from kinetic properties of binding among specific probes and targets at saturation (24). The third and probably the most important issue for compromising informational efficiency in cDNA-based array is the domain of image acquisition and image analysis. As discussed elsewhere, these processes often include computation based on arbitrarily chosen parameters, set points and cut offs, especially in deciding the balance between signal and noise, pixel characteristics and normalization strategies (25). Insufficient application of statistical inference may also skew the evaluation of estimates retrieved from cDNA-based array analysis (26).

**Physiological issue**

It is generally known that human placental growth during 4- to 8-weeks of gestation takes place in an exponential manner and is critical for subsequent placental function and fetal development (27, 28). We now report that categorical expression of four genes (CXCR4, ERBB2, HDAC1 and TNFRSF1A) and general expression of two genes (COL4A4 and HPRT1) are associated with the development of placental villi during 6- to 8-weeks of gestation.

Table V shows the gene ontology based descriptions of the six candidate gene products examined in the present study.

It appears evident that chemokine (C-X-C motif) receptor 4 (CXCR4) and tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) are directly involved in inflammatory responses in mammalian tissues (see table 5). Furthermore, CXCR4 is involved in chemotaxis, macrophage activation and blastocyst implantation (29-31). On the other hand, TNFRSF1A member protein interact with various intracellular signaling molecules involved in the regulation of apoptosis (32). Collectively, it appears that timed regulation of these two candidate gene products in reciprocal manner supports categorically the developmental transition of placental villi from growth phase to differentiation phase between 6- and 8-weeks of gestation.

Interestingly, two candidate gene products (ERBB2 and HDAC1) showed categorical up-regulation predominantly between 6- and 7-weeks, and 7- and 8-weeks of gestation, respectively. We believe the time profile of the expression of these two gene products are important in view of the facts that (i) ERBB2 (erythroblastosis oncogene B2) protein is proto-oncogenic in nature, and it encodes for epidermal growth factor receptor and supports cell proliferation (33), and (ii) the product of HDAC1 (histone deacetylase 1) gene plays key role in the regulation of eukaryotic gene regulation by histone modification (34).

The housekeeping activities of COL4A4 and HPRT1 are maintained in placental villi throughout 6- to 8-weeks of gestation. COL4A4 encodes collagen alpha 4 (IV) chain protein which is integral to extracellular matrix and basement membrane constituent (35). On the other hand, HPRT1 encodes for an enzyme – hypoxanthine phosphoribosyltransferase 1 – involved in recycling purines via salvage pathway and thereby DNA and RNA production
Clearly, the sustained activities of these gene products are required for continuous growth and differentiation of the tissue. The deficiencies of these factors are known to result in several genetic disorders (37, 38).

In conclusion, we infer that:

(i) cDNA array-based data are to be verified in terms of quantitative estimates preferably by quantitative polymerase chain reaction before these
are used for any paradigmatic manner for biomedical purposes.

(ii) categorical regulation of genes responsible for cell proliferation and differentiation, for example ERBB2 and HDAC1, in one hand, and that of genes responsible for inflammatory responses and apoptosis, for example CXCR4 and TNFRSF1A, on the other hand are required to balance for normal development of placental villi during 6-8 weeks of gestation. Sustained housekeeping activities of genes, for example COL4A4 and HPRT1, are integral to the early stage developmental program of human placental villi.

(iii) the present report documents for the first time the potential marker functions of the six genes, namely COL4A4, CXCR4, ERBB2, HDAC1, HPRT1 and TNFRSF1A, in the normal process of the early developmental program in human placental villi.

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