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Research Article

Molecular Characterization of the Vasculature of Patients with Infiltrating Ductal Carcinoma Generates a Gene Signature Predictive of Breast Cancer Survival

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Abstract

Targeting of the tumor vasculature has evolved into an integral part of existing standard anti-cancer therapies. However, recent pre-clinical and clinical studies have shown that the efficacy of these treatments is often temporary and frequently followed by renewed tumor growth. In an attempt to overcome this problem we transcriptionally profiled laser capture micro dissected micro vessels from both tumor and matching adjacent normal breast tissue obtained from patients diagnosed with infiltrating ductal carcinoma (IDC). Vascular enrichment of the LCM samples was confirmed by Q-PCR of CD31 and transcription profiles were generated and analyzed using the Genespring software and the WebGestalt Toolkit. Hierarchical two-dimensional clustering of the transcriptome data identified 219 significantly up-regulated transcripts in at least 4/8 patient samples (cut-off >1.3-fold, $p<0.05$). Several of these genes (i.e. AGRN, FLNA and ILR4) were selected and their over expression was confirmed at the protein level in tumor versus normal tissue. However, the overall heterogeneity we observed in the vascular gene expression patterns pose a significant problem for individual genes/proteins to be used as a robust breast cancer vascular specific marker or validated therapeutic target. We therefore mined our data set further by applying an in-house developed bioinformatics network method incorporating clinical information of the patient. This approach generated a 15-gene 'Vascular-Derived Prognostic Predictor' which, using six publicly available datasets, robustly identified those patients with an increased risk of recurrence.

ABBREVIATIONS

LCM: Laser Capture Micro Dissection; IDC: Infiltrating Ductal Carcinoma; OCT: Optimal Cutting Temperature; VDPP: Vascular-Derived Prognostic Predictor; IHC: Immunohistochemistry.

INTRODUCTION

Worldwide, breast cancer still has the highest morbidity and mortality in the Western world [1]. The past decade has nonetheless seen a tremendous improvement in our understanding of the mechanisms underlying this disease. This has not only translated in the development of new and better therapeutics but also in the identification better diagnostic and predictive molecular markers.

During the last 10 years it has become evident that the microenvironment plays a pivotal role in tumor development. This microenvironment consists of normal cells, connective tissue, inflammatory cells and also micro vessels, which provide oxygen and nutrients, and which function as a waste removal

system for the developing tumor. Most tumors initially grow avascularly, which allows the tumor to persist in a dormant state, with only a small number of tumors developing into a more vascular dependent state. Tumor angiogenesis has been identified as one of the hallmarks of cancer [2,3], and correlates with tumor aggressiveness, metastasis, and poor patient outcome [4]. Under normal physiological conditions, angiogenesis is a tightly controlled balance of numerous mechanisms involved in anti- and pro-angiogenic events [5]. However, in a developing tumor, genetic mutations, mechanical stress, and processes such as inflammation and hypoxia [6], can tip this balance in favor of pro-angiogenesis [7,8]. This creates an environment that is favorable for uncontrolled and abnormal micro vessel formation [9-11]. As a result, the tumor vasculature is often an abundant architectural chaos of irregular shaped dilated, tortuous, frequently leaky and hemorrhagic blood vessels. It is for this reason that tumors are often referred to as 'wounds that never heal'.

Because of its role in tumor development, the tumor vasculature has become an attractive therapeutic target, with many attempts having been made to harness tumor growth by inhibiting or by normalizing tumor angiogenesis. For example Bevacizumab, a monoclonal antibody targeting VEGF-A [12], is used in combination with standard chemotherapy, while Sunitinib and Imatinib (both small molecule receptor tyrosine kinase inhibitors) are currently used as mono therapy [13-17]. Nonetheless, and despite initial promising results, Bevacizumab failed to improve the overall survival of patients. One possible explanation is that tumors can adapt and escape anti-angiogenic treatment by switching to alternative angiogenic pathways, such as vessel co-option [18], intussusceptive angiogenesis [19], recruitment of endothelial precursor cells [20], and vasculogenic mimicry [21]. These observations suggest that targeting the VEGF pathway may actually promote instead of halting the invasion and metastasis of tumor cells [22,23].

Several efforts have been undertaken to identify better angiogenic therapeutic targets or biomarkers by evaluating the gene expression patterns of endothelial cell lines [24,25], and of endothelial cells isolated from fresh tissues via enzymatic digestion [26,27]or from frozen [28-32] or fixed [33,34] tumor tissues via laser capture microdissection (LCM). Since LCM has the advantage of keeping the integrity of the genetic material intact, we used this approach to isolate endothelial cells from tumor and matching normal clinical breast tissue samples obtained from ten patients diagnosed with infiltrating ductal carcinoma (IDC) and performed a proteomic (Published by Hill *et al.*, [30]) and a transcriptomic (described here and outlined in Figure 1) analysis. Although individual prognostic biomarkers may help to predict disease outcome, they often have poor predictive power. However, the use of biomarkers sets derived from genome-wide expression profile scan overcome this problem [35-39]. With the goal of identifying prognostic biomarker sets, we applied an in-house developed integrative network approach [40,41] which integrated the clinical data from the patient samples with the vascular specific transcriptome read-out of each sample. This approach, which generated a 15-gene 'Vascular-Derived Prognostic Predictor' (VDPP), demonstrated a robust survival prognostic power (P-values <0.05) in six independent publicly available patient cohorts [42-47]. The results presented here

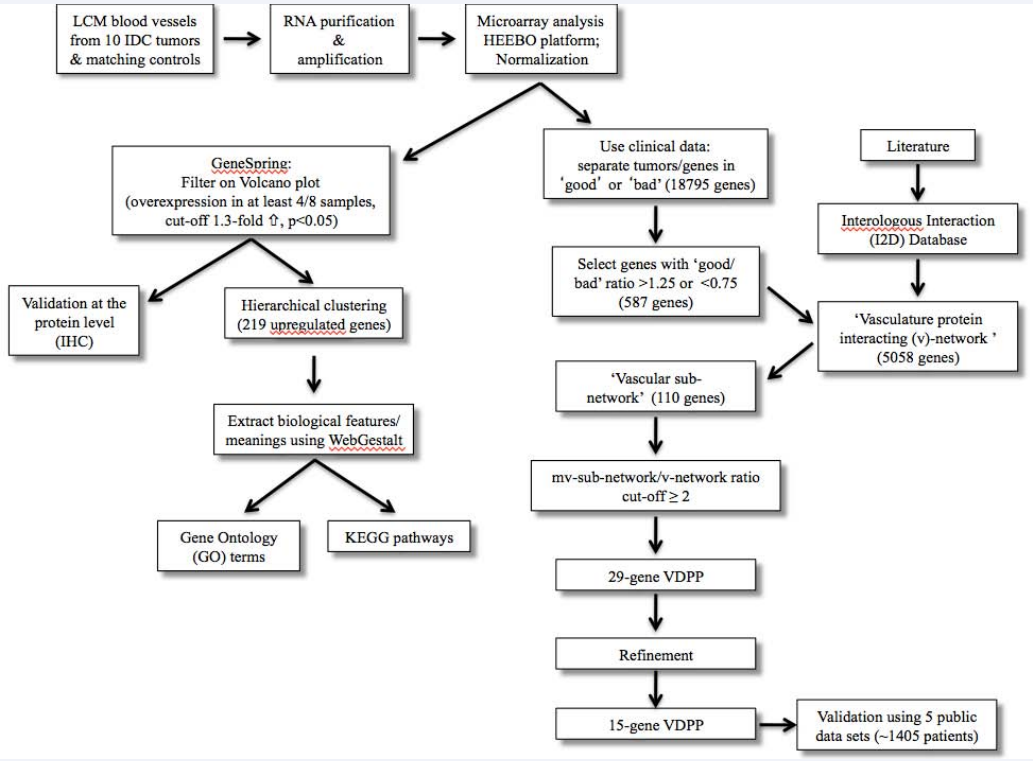

Figure 1 Flow chart outlining the principal steps in the generation, analysis, and validation of the gene expression data set from microvessels isolated by LCM from clinical IDC samples (see text for details).

clearly show that this vascular-derived signature can be used to identify cohorts of patients that are at high risk versus low risk for local cancer recurrence [35-39].

MATERIALS AND METHODS

Tissue preparation and sectioning

Ten (10) frozen human tumor specimens and their matching adjacent non-tumor tissues from patients diagnosed with breast cancer were obtained from the Ontario Tumor Bank (OTB; Toronto, ON, Canada). Specimens were selected using the following criteria: 1) gender (female), 2) histology (infiltrating ductal carcinoma, IDC), 3) availability of normal adjacent breast tissue, 4) high degree of vascularity, 5) availability of clinical patient data, and 6) patient age (between 35 and 65 years). Frozen tissue samples were embedded in OCT, cut (at -21°C) into 8 µm serial sections using a cryotome (Leica CM1900, Richmond Hill, ON, Canada), mounted on Super frost Plus microscope slides (Fisher Scientific, Ottawa, ON, Canada), and kept at -80°C until used. The Human Ethics Committee of the OTB and National Research Council of Canada approved this study and all human subjects provided a written, informed consent.

Microvessel staining and LCM capture

Microvessels in both normal and tumor tissue sections were stained with fluorescein-labeled UlexEuropeaus Agglutinin I lectin (UEA1; Vector Laboratories, Burlington, ON, Canada) as described previously [48]. UEA1-labeled blood vessels were dissected using a PixCell II-LCM instrument (Molecular Devices, Sunnyvale, CA, USA) with a 7.5 µm laser spot size, 0.75-0.90 ms laser pulse, and a 25-30 mV laser beam power. Approximately 500-700 laser shots (microvessel cells) were captured per high-sensitivity (HS) cap (CapSure HS LCM Caps; Molecular Devices, Sunnyvale, CA, USA). Two to three caps containing a total of 1,000-1,500 shots were collected per tissue sample, lysed in 100 µL of RNA lysis buffer containing 0.7 µL of β-mercaptoethanol (Stratagene, La Jolla, CA, USA) and used for RNA isolation, amplification, and labeling prior to microarray analyses.

Real-time PCR (Q-PCR)

Total RNA was extracted from both LCM captured material and wholemount frozen sections of both tumor and matching normal tissue sample using a Tissue Ruptor with disposable probes (Qiagen Inc., Toronto, ON, Canada) and a RNeasy Plus Mini-Kit including a DNase step (Qiagen Inc., Toronto, ON, Canada). RNA quality and integrity were assessed using an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, Mississauga, ON, Canada). cDNA was prepared using Super Script II RT in combination with random primers (both Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. Q-PCR was performed using the Mx3005P QPCR System (Agilent Technologies, Mississauga, ON, Canada) and a QuantiTect SYBR Green PCR master mix (Qiagen Inc., Toronto, ON, Canada). For the amplification of 28S (Fwd primer: TTGAAAATCCGGGGGAGAG; Rev primer: ACATTGTTCCAACATGCCAG) and CD31 mRNA (Fwd primer: AACAGTGTTGACATGAAGAGCC; Rev primer: TGTAAAACAGCACGTCATCCTT), samples were heated for 10 minutes at 95°C followed by 40 amplification cycles (95°C, 30 s; 55°C, 30 s; 72°C, 30 s). Enrichment of the CD31 content in normal and tumor LCM samples compared to wholemount slices was calculated using the ΔΔCt method as described (SABiosciences, Frederick, MD, USA).

RNA isolation, amplification and labeling

Total endothelial RNA was extracted according to the manufacturer's instructions (PicoPure RNA Isolation Kit; Molecular Devices, Sunnyvale, CA, USA), and further amplified using the HS RNA Amplification Kit (Arcturus, Mountain View, CA, USA) according to the manufacturer's instructions. The generated antisense (a)RNA was then amplified and labeled with either Cy3 or Cy5 mono functional dye (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using the Amino AllylMessageAmpTM II aRNA Amplification kit (Ambion, Austin, TX, USA) according to he manufacturer's instructions. RNA purity (A260/280 ratio) and Amino Allyl incorporation levels (A289/260 ratio) were determined using the Bio-analyzer (Agilent Technologies, Mississauga, ON, Canada).

Microarray analysis

Labeled and purified vascular aRNA was hybridized to an in-house printed microarray chip containing 48,958 70-mer Human Exonic Evidence Based Oligonucleotide probes (HEEBO; Invitrogen, Burlington, ON, Canada). A total of 4 hybridizations, including technical repeats and dye-swap hybridizations, were carried out for each of the patient samples. Slides were scanned using a Scanarray 5000 dual-color confocal laser scanner (Perkin-Elmer, Waltham, MA, USA) at 535 nm (Cy3) and 635 nm (Cy5) and fluorescent images were quantified using the QuantArray software package (Perkin-Elmer, Waltham, MA, USA). Data normalization (Lowess algorithm) and hierarchical clustering were performed as described using GeneSpring GX v7.3 (Agilent Technologies, Santa Clara, CA, USA) [49]. Significantly up-regulated genes were selected by 'Filtering on Volcano Plot' while applying the following criteria: 1.3-fold variation in a minimum of 4/8 patients with $P < 0.05$.

Immunohistochemistry

Frozen tissues were sliced using a cryotome (Leica CM1900, Richmond Hill, ON, Canada) at a thickness of 6 microns and air dried (5 min, RT) prior to fixation (10% buffered-formalin, 5 min). Endogenous peroxidase was quenched (3% H_2O_2 solution), and non-specific sites were blocked using Ultra V Block (Labvison; Thermo Fisher Scientific Inc., Fremont, CA, USA). This was followed by an incubation with either mouse specific anti-CD31 (BBA7 clone 9G11; R&D Systems, Burlington, ON, Canada; 1:200, 2 hr, RT), anti-Agrin (MAB458; Chemicon Millipore, Billerica, MA, USA; 1:20, 2 hr, RT), anti-FLNA (ab3261 clone PM6317; Abcam, Cambridge, MA, USA; 1:600, 1 hr, RT), or anti-IL4R (MAB230; R&D Systems, Burlington, ON, Canada; 1:100, 2 hr, RT). Slides were rinsed with buffer (TBS-0.1% Tween 20) after this and in between each of the following steps. Tissues were incubated (30 min at RT) with secondary antibody (HRP-polymer-conjugated antibody UVone reagent; Thermo Fisher Scientific Inc., Fremont, CA, USA), while AEC reagent (Thermo Fisher Scientific Inc., Fremont, CA, USA) was used as HRP substrate. Sections were counterstained with Hematoxylin 560 (Surgipath Winnipeg, MB, Canada), prior to mounting (Aquatex; Surgipath Winnipeg, MB, Canada) and microscopic evaluation.

Functional annotation

Gene ID Converter (http://idconverter.bioinfo.cnio.es/IDconverter.php) was used to generate EntrezGene IDs of the 219 up-regulated genes, these IDs were then loaded into the WebGestalt software (Web-based Gene SeTAnaLysis Toolkit, http://bioinfo.vanderbilt.edu/wg2/) [50]. Genes were categorized in three categories ('Cellular Component', 'Molecular Function', and 'Biological Process') using the GO Slim tool and the gene set was further evaluated for enrichment in the KEGG biochemical pathways (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.ad.jp/kegg) [51].

Bioinformatics network analysis

By using the associated clinical data, the normalized microarray gene expression data for each patient was classified as either 'good' (without local recurrence) or 'bad' (with local recurrence). For each of these genes, the 'good' versus 'bad' ratio was calculated, and genes with ratios below 0.75 and over 1.25 were assumed 'modulated genes'. Genes involved in vascular development were identified using Gene Ontology (GO) annotations and the literature searches [52,53], which allowed for construction of a vascular development centered protein network (v-network) by using these genes and their directly interacting neighbors in a human protein network that was downloaded from the Interologous Interaction (I2D) Database (http://ophid.utoronto.ca/ophidv2.201/) . Modulated genes were then filtered on this v-network and only those present in the v-network were then used to generate a so-called 'vascular-modulated' (vm)-sub-network. Genes on this vm-sub-network were then ranked based on their connectivity (i.e. the number of links a gene has with other genes), which was calculated as the ratio of the number of connections in the mv-sub-network versus those in the v-network. The resulting ranked 29 gene set was then validated for its predictive power using a training data set [42], and was further refined by removing genes 'one-by-one-bottom-up' until a significant increase in P-value was observed. The prognostic power of the gene sets was evaluated using a Kaplan-Meier analysis which implements the Cox_Mantellogrank test using the statistical computing language R (http://www.r-project.org/) [40,41]. The resulting final 15-gene set was tested in an additional five independent publicly available data sets of solid breast tumors [43-47].

RESULTS AND DISCUSSION

LCM capture of endothelial cells

To analyze the differences in vascular gene expression patterns in the normal and tumor breast vasculature, we used LCM-captured microvessels from tumor and matching normal samples of breast cancer patients diagnosed with IDC. Eight µm thick tissue sections were stained with fluorescein-labeled UEA1, a lectin that specifically binds to the fucose residues of glycoproteins or glycolipids present on the cell surface of endothelial cells [54]. This staining method allowed for an easy identification (Figure 2A) and capturing (Figure 2B) of the blood vessels, and also demonstrated the higher density and more tortuous appearance of these microvessels in the IDC samples. To ensure optimal quality, the aRNA integrity was verified after amplification and labeling (see Materials & Methods), and only aRNA samples with an A260/280 ratio (purity) of between 2.0-2.6 and an A289/260 ratio (Amino Allyl incorporation level) of between 0.2-0.3 were used for the microarray study. In general, good quality aRNA appeared as a broad peak, ranging from 200 to 2000 bases in length. Using these criteria we excluded two of the ten patients (patient #299 and #314) from the transcriptome analysis.

Real-time PCR (Q-PCR)

To ensure endothelial enrichment in the LCM-captured material, we evaluated the CD31 levels in the LCM samples (normal and tumor) and compared these to those in the matching

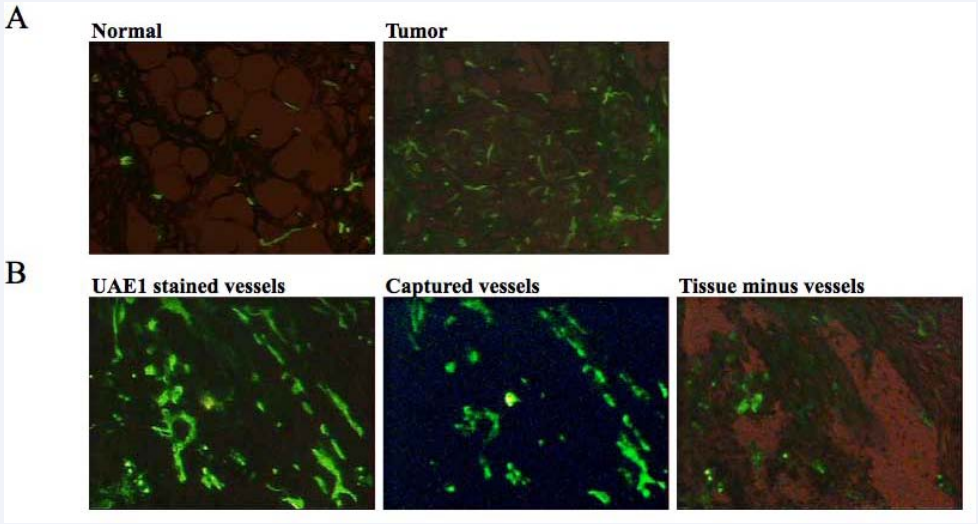

Figure 2 Isolation of microvessels by LCM. (A) Microscopic view (Magn. 40x) of microvessels stained with fluorescein-labeled UEA1 (green) in clinical samples of patients diagnosed with IDC (right side); the patient's matching normal tissue is shown on the left. (B) Identification of UEA1-labeled microvessels in the IDC samples (right panel) isolated by LCM. The middle panel shows the same section from which the microvessels were removed, while the right panel shows the isolated microvessels on the HS cap.

whole-mount sections by Q-PCR. CD31 is an endothelial cell specific adhesion molecule and is generally used as a marker for normal and neoplastic vascularization. Using human CD31 specific primers and after normalization (28S mRNA), we confirmed the endothelial enrichment in the LCM material extracted from the normal and tumor tissues (an 8.5- and 17.6-fold increase in CD31 transcript levels, respectively (data not shown)).

Differential Gene Expression Between LCM-Captured Vessels from IDC and matching normal breast tissues

To identify genes that are specifically up-regulated in the tumor microvasculature, we interrogated the transcriptome of the LCM dissected IDC microvessels of the selected eight patients, using microarray chips that were printed in-house with the HEEBO oligo probe sets. In order to eliminate genetic background and other differences between the individual patient samples, we choose to co-hybridize RNA isolated from the tumor and normal microvessels in an intra-patient manner. Since our goal was to identify novel vascular specific biomarkers or therapeutic targets, we used the 'selection on volcano plot' option in the GeneSpring software with an expression level cut-off of 1.3-fold and $p<0.05$, and selected 219 genes that were up-regulated in at least 4/8 patients (Supplemental Table S1). Two-dimensional hierarchical clustering [55] of these genes (Figure 3) showed that the vascular expression patterns of these eight patients differs significantly (horizontal dendrograms), which agrees with earlier reports[32]. Nonetheless, several of these up-regulated genes are associated with vascular development, such as von Willebrand factor and the endothelial Pas domain protein 1.

Functional annotation

Of the 219 up-regulated transcripts, Gene ID Converter was able to retrieve 140 EntrezGene IDs which were then loaded in the Web-based Gene SeTAnaLysis Toolkit (WebGestalt) software [50] to extract biological features and meanings. Using the Gene Ontology [56] (GO) Slim classification tool, genes were categorized under 'Cellular Component', 'Molecular Function', and 'Biological Process' (hypergeometric statistical method, Benjamini and Hochberg multiple test adjustment, 'Top 10' significance level). An overview of this analysis can be found in Supplemental Figure S1. An additional enrichment analysis using the KEGG pathways, showed that, with a minimum number of four genes for a category, up-regulated genes could be found in Jak-STAT, MAPK, and PPAR signaling, in cytokine-cytokine receptor and neuroactive ligand-receptor interactions, in focal adhesions, and in metabolic and cancer pathways (data not shown).

Validation of gene expression by immunohistochemistry

We then picked three of the top genes (Agrin (AGRN), Filamin A (FLNA), and Interleukin-4 receptor (IL4R)), which are overexpressed in the microvasculature of all eight patients (Supplemental TableS1).Validation was carried out at the protein level by immunohistochemistry (IHC) in both normal and tumor sections of all eight patients used in the micro array study. In addition, two independent patient samples not used in the transcriptome analysis were selected based on the same criteria used for choosing the LCM/microarray analysis samples

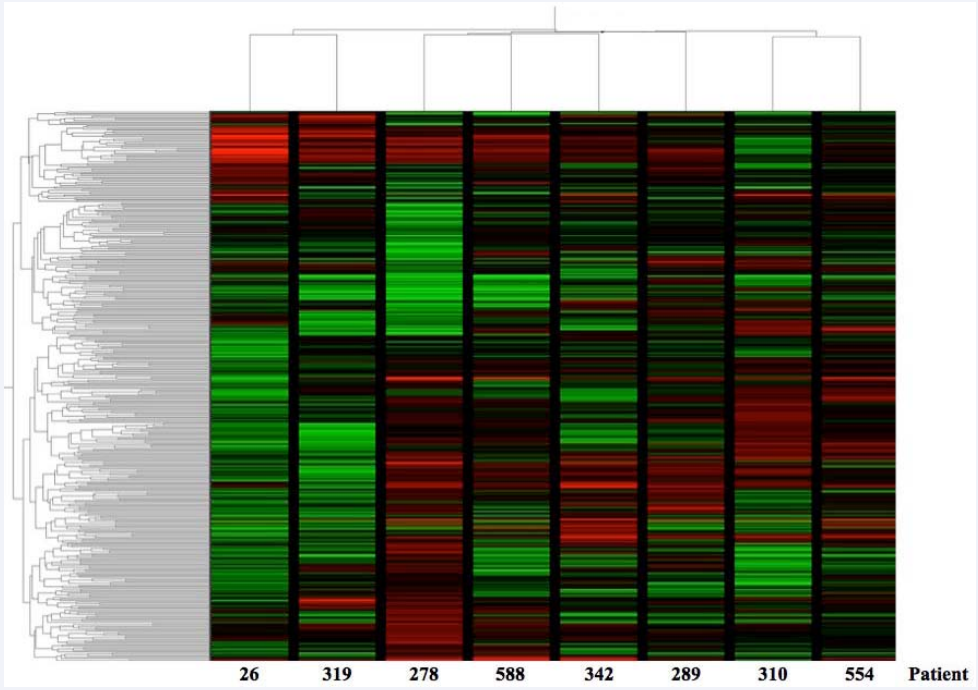

Figure 3 cDNA microarray analysis of the LCM isolated microvessels obtained from eight patients diagnosed with IDC. Two-dimensional hierarchical clustering of statistically significant modulated genes in the tumor microvasculature (up-regulated, red; down-regulated, green) that were identified using 'Filtering on Volcano plot'. A total of 219 genes (Supplemental Table S1) that were up-regulated in at least 4/8 patients with a 1.3-fold cut-off ($P<0.05$) were selected. Vertical dendrograms illustrate similarities between gene expression profiles; horizontal dendrograms show similarities between the eight patients (labeled with 'patient number'). Up-regulated gene set (Supplemental Table S1) was further evaluated for the enrichment of biological processes using the web-based WebGestalt Toolkit (Supplemental Figure S1).

(see Materials & Methods). Figure 4 shows the stained tissue sections of two representative patients; patient #588 (A), which was included in the LCM/transcriptome study, and patient #354 (B), which was not included in the transcriptome study. CD31 staining was used to identify the vasculature and clearly shows distinct blood vessels in the normal tissues and the vascular chaos in the tumor tissue. The staining of the tissue of these two patients illustrates differential expression in tumor versus normal tissue, i.e. all three proteins were strongly stained in tumor tissue while AGRN andIL4R was essentially undetectable in normal tissue, and FLNA was low in normal tissue. Similar observations were made for the other patient samples that were analyzed (data not shown). Based on these staining patterns, it is apparent that these proteins do not specifically co-localize only with the vasculature, e.g. in patient #588 FLNA is obviously expressed throughout the tumor. To examine co-localization further, we used immune fluorescent microscopy, which also indicated that the expression of these proteins was not restricted to the tumor microvasculature (data not shown).Variability in protein expression and pattern between patient samples was observed (see Figure 4). This likely reflects the heterogeneity found in breast cancer tumor samples in general, and makes it extremely difficult to use single proteins as biomarkers and to select relevant therapeutic targets.

Identification of a 15-gene vascular-derived prognostic predictor

Since tumor heterogeneity made it difficult to identify robust microvascular-specific biomarkers from our transcriptome and IHC data alone, we analyzed our data set further by using a bioinformatics network approach that incorporates the clinical (survival) data (5 year and more) of these patient samples. Sufficient clinical data was available for six of the eight patients (#26, #278, #289, #310, #319 and #342) which allowed for the respective tumor vascular samples to be divided into two groups representing patients with either a 'good outcome', i.e. without local recurrence (patient #26, #278, #289 and #310) or 'bad outcome', i.e. with local recurrence (patient #319 and #342). We identified all genes that were modulated (up- or down-regulated) between these two groups and calculated 'good'/'bad' outcome ratio (Figure 5A). A total of 587 genes whose expression ratio was either >1.25 (369 genes) or <0.75 (218 genes) was identified. Using a 'vasculature protein interacting (v)-network', which was generated from the literature and consisted of 5058 proteins centered or involved in the molecular mechanisms that underlie vasculature development, we then filtered the selected 587 vascular genes onto this v-network. This filtering step generated a smaller 'modulated vascular' (mv)-sub-network

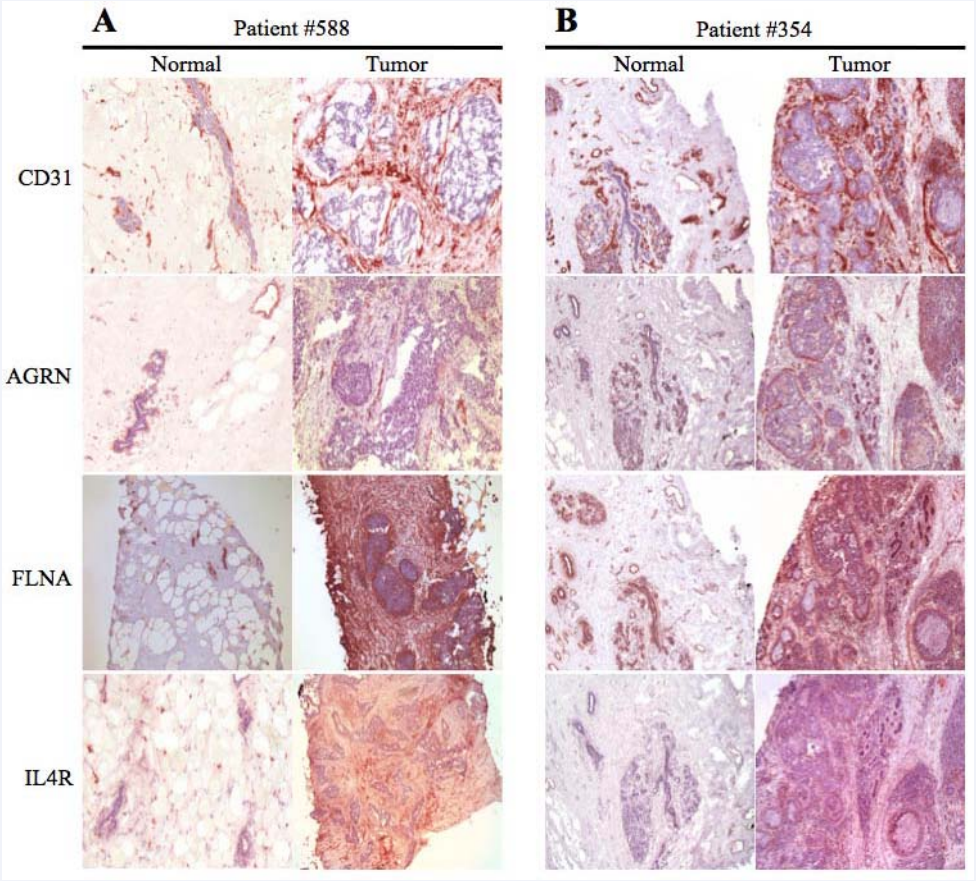

Figure 4 Validation of selected genes at the protein level. Sequential sections of normal (left panels) and tumor (right panels) samples obtained from (A) patient #588 (used for the LCM microarray analysis) and (B) patient #354 (independent patient sample selected using the same criteria as patient #588) were cut and stained for CD31 (identification of blood vessels), and for Agrin (AGRN), Filamin A (FLNA), and Interleukin-4 receptor (IL4R).

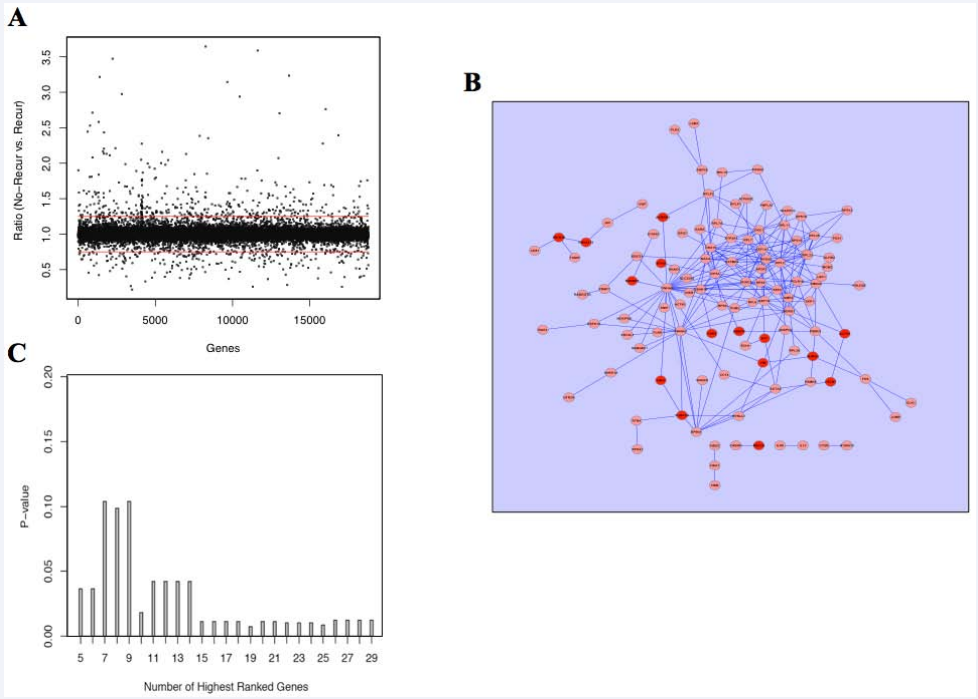

Figure 5 Analysis of the gene expression data using an in-house developed systems biology network approach. (A) Using clinical data available for 6/8 patient samples, four tumor samples were classified as 'good' (patients without recurrence) and two as 'bad' (patients with recurrence); a total of 18795 genes were found to be modulated between these two groups. The 'good'/'bad' ratio for each of these genes was then calculated and a total of 587 genes whose ratio value was <0.75 or >1.25 (cut-offs are indicated by the red lines), were selected for further analysis (see text for details). (B) Mv-sub-network (for details see text) consisting of 110 genes that were modulated between 'good' and 'bad' tumors and that were involved in, and/or closely related to vascular development. (C) Twenty-nine genes whose mv/v ratio was ≥ 2 were then ranked (see text for details). 'One-by-one-bottom-up' removal of genes did not change the predictive P-value (calculated using the Chang data [42] as a training set) of the VDPP until the 15th gene was removed. VDPP genes are shown as dark-red solid circles in the mv-sub-network (Figure 6B) and are listed in Table 1.

(Figure 5B), which consists of 110 genes that are involved in and/or closely related to vascular development and whose expression levels differ significantly between the vasculature in patient tumor samples with and without local recurrence. In order to determine which of these genes are more critical to vascular development, we then assessed the number of connections of each of these 110 genes with other genes in both in the mv-sub-network and v-network. Genes were then ranked according to the calculated mv-sub-network/v-network ratio, which, according to the network theory [57,58], reflects a gene's chance to be involved in the transcript level modulations that are observed between 'good' and 'bad' tumor samples. Finally, by applying an mv-sub-network/v-network ratio cut-off ≥ 2, a total 29 genes were selected. This signature was further refined by removing genes 'one-by-one-bottom-up' followed by retesting of this shortened list using Chang's data [42] as training set. This iterative process showed that the predictive P-value of the gene signature remained relatively unaffected until the 15th gene is removed (Figure 5C). Lastly, we validated this 15-gene vascular-derived prognostic predictor (VDPP; shown as red filled circles in Figure 5B, listed in Table 1) using five additional publicly available solid breast tumor data sets [43-47]. The results of this analysis demonstrates that this VDPP is able to identify patients that are at higher and lower risk of local recurrence within the tested patient populations (Figure 6) with a robust predictive power (P-values for all data sets <0.05).

CONCLUSION

The data presented here is the second part of a larger study in which we used either a transcriptome analysis (presented here) or proteomic analysis approach (published by Hill *et al.*[30]), to identify new vascular biomarkers and/or therapeutic targets involved in the regulation, maintenance and growth of the tumor microvasculature in samples from breast cancer patients diagnosed with infiltrating ductal carcinoma (IDC). Both studies isolated microvessels from the same set of frozen IDC and matching patient control samples (Figure 2), using an LCM approach that was guided by the labeling the vasculature with fluorescein-labeled UlexEuropeaues Agglutinin I (UEA1) [59,60]. This method, which was successfully used for the LCM isolation of microvessels from clinical glioblastomamultiforme (GBM) by Pen *et al.* [31], not only allows for the isolation of blood vessels from their natural environment but also has the advantage that pericytes, which play an important role in (tumor) microvessel maturation [61-63], are included.

Following confirmation of the enrichment of normal and tumor vasculature in the LCM samples by real time Q-PCR detecting the endothelial CD31 transcript, interrogation of the transcriptome was carried out with the RNA isolated from 8patients. We then used Genespring to select a total of 219 genes that were up-regulated>1.3-fold (p <0.05) in the vasculature of at least 4/8 IDC samples. To gain some insight into the biological

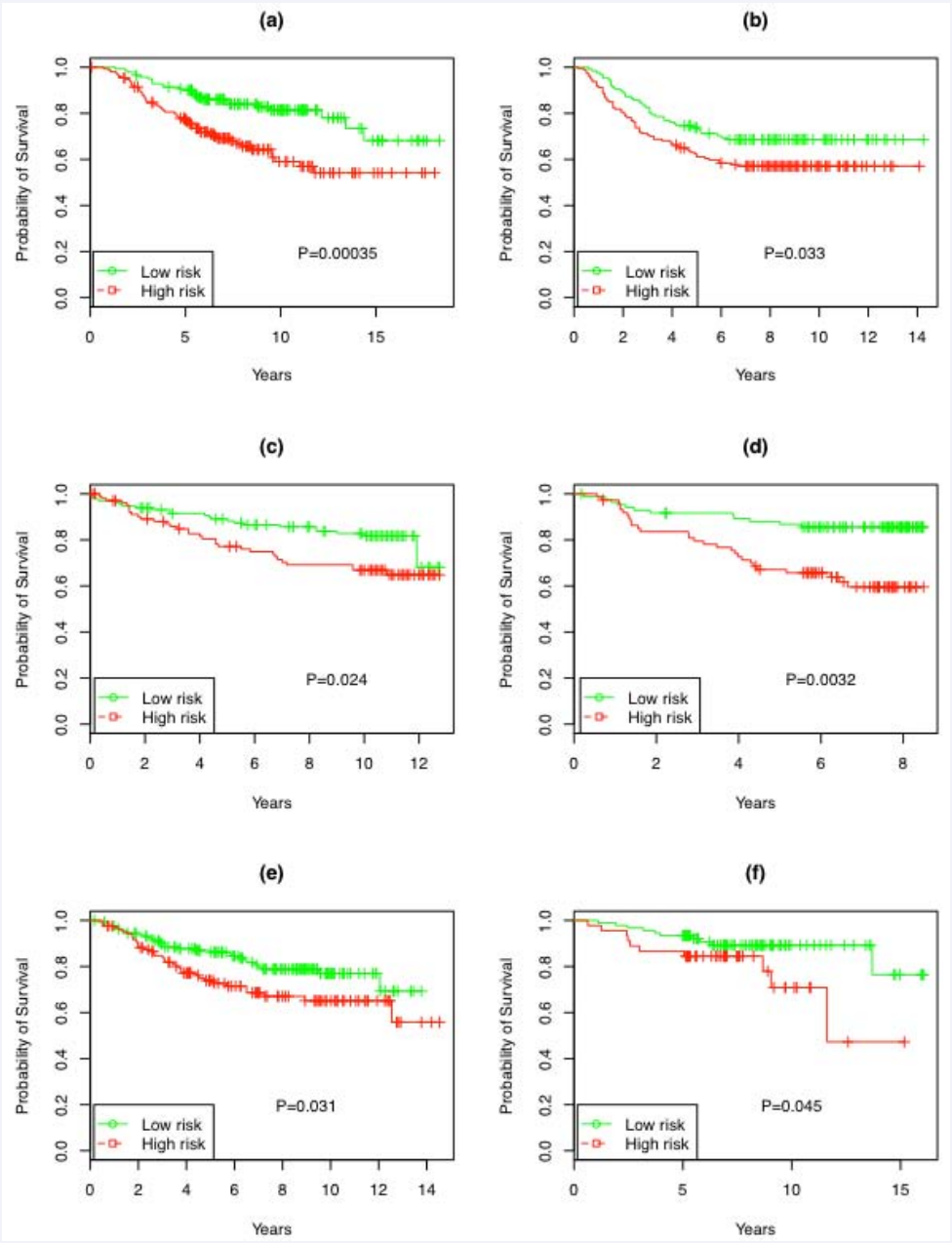

Figure 6 Kaplan-Meyer curves of the risk groups for local tumor recurrence in breast cancer patients predicted by using our 15-gene VDPP. (A) Chang cohort [42], (B) Wang cohort [46], (C) Miller cohort [44], (D) Pawitan cohort [45], (E) Loi cohort [43], and (F) Zhang cohort [47] containing data from 295, 286, 236, 159, 293, and 136 patients respectively (red curves, high-risk groups; green curves, low-risk groups). P-values were obtained using the χ2-test.

processes in which the up-regulated genes are involved we used the web-based WebGestalt Toolkit (http://bioinfo.vanderbilt.edu/wg2/) [50]. Based on GO terms, most of the up-regulated genes we identified are involved in metabolic processes and are membrane-bound (Supplemental Figure S1).

From our gene list we selected three genes that were highly over expressed in all tumor samples and for which IHC suitable antibodies were available (see Supplemental TableS1, marked yellow). Agrin (AGRN) is one of three main basement membrane heparan sulfate proteoglycans (HSPGs) reported to modulate cancer growth and angiogenesis [64], whereas the expression of Filamin A (FLNA) in combination with VEGF has been shown to promote lung tumor development [65]. The interleukin-4 receptor (IL4R) is an important molecule in B and T cell development, but has also been reported to play an important role in tumor progression [32,40]. IHC evaluation of CD31, AGRN, HSPG and FLNA protein expression in sections from both tumor and normal tissue from eight patient samples confirmed that AGRN, HSPG and FLNAwere generally expressed at higher levels in tumor tissue relative to normal tissue. However, the staining also revealed a high degree of intra-tumor heterogeneity and

inter-patient variability in both expression levels and patterns. Figure 4 illustrates: 1) the generally higher level of expression of AGRN, HSPG and FLNA in tumor versus normal tissue, 2) heterogeneous expression within the tumor tissue from a single patient and variability between patients, and 3) that expression of these proteins is not confined to the vasculature alone (i.e. not restricted co-localization with CD31). This latter observation is not totally unexpected since tumors have been reported to exhibit 'vasculogenic mimicry', which involves the transdifferentiation of aggressive tumor cells into cells expressing endothelial cell and pericyte features [66]. This transdifferentiation results in structures that resemble embryonic vascular networks [67]. Vasculogenic mimicry was first reported for aggressive melanoma cells [68] but has also been observed in several other tumor types including breast, lung, prostate and ovarian carcinomas[69]. Although we confirmed vascular enrichment of our LCM dissected samples which we used for transcriptome analysis, it may be that the vascular LCM samples also contained tumor cells which had undergone vasculogenic mimicry.

Endothelial cells are considered to be a good therapeutic target since they are thought to be genetically very stable [70]. Nonetheless, the clinical benefits from anti-angiogenic therapies have been limited due to drug resistance [71], and possibly also microvessel heterogeneity [72]. Despite the fact that we selected patient samples using the same criteria, and that we attempted to reduce variability by hybridizing microarray chips with tumor and normal vascular-derived RNA in an intra-patient manner, we observed different overall vascular expression patterns for each of the eight patients (Figure 3). Various studies have reported on differential gene expression patterns that are characteristic of the tumor vasculature by comparing normal and tumor-associated blood vessels [26-29,31,73,74]. Additionally, a recent publication of Pepin *et al.* (2012) specifically addressed tumor vasculature heterogeneity [75]. These publications and the results we present here indicate that it will be extremely difficult to identify a single tumor vascular specific biomarker.

We therefore sought to further explore our data set by using a more global bioinformatics network analysis. We successfully used this type of analysis in the past and developed an algorithm that can be used to classify breast cancer patients as either high- or low-risk groups in terms of recurrence [41]. Applying a similar approach to the list of differentially expressed IDC specific vascular genes, we generated a 'Vascular-Derived Prognostic Predictor (VDPP)' (Figure 5). This transcriptome-derived VDPP shows however no overlap with the 29-protein-based predictive signature that was published earlier by Hill *et al.*[30]. This is not surprising since transcriptome and proteome datasets usually show only a weak positive correlation due to e.g. differences in RNA and protein processing, and protein degradation processes [76, 77]. These functional differences may however provide new insights into the regulation of neovascularization and microvascular maintenance in the tumor.

Using 6 independent publicly available datasets (totaling 1405 patients) [42-47]we showed that the transcriptome-derived VDPP signature stratifies patient cohorts (with a predictive P-value < 0.05) into groups with a high- and low- risk of local recurrence after surgical removal of the tumor (Figure 6). It has been reported that 70-80% of lymph-node negative patients may undergo unnecessary chemotherapy [78], and that for a large number of early stage breast cancer patients chemotherapy may not be beneficial [35]. Use of the VDPP we described here may thus help in the identification of breast cancer patients with a low risk for local recurrence, which are not likely to benefit from adjuvant chemotherapy treatment and thereby unnecessarily suffer the side effects associated with c hemotherapy.

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