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Research Paper

Radiogenomics Monitoring in Breast Cancer Identifies Metabolism and Immune Checkpoints as Early Actionable Mechanisms of Resistance to Anti-angiogenic Treatment

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ABSTRACT

Anti-VEGF antibody bevacizumab has prolonged progression-free survival in several cancer types, however acquired resistance is common. Adaption has been observed pre-clinically, but no human study has shown timing and genes involved, enabling formulation of new clinical paradigms.

In a window-of-opportunity study in 35 ductal breast cancer patients for 2 weeks prior to neoadjuvant chemotherapy, we monitored bevacizumab response by Dynamic Contrast-Enhanced Magnetic Resonance [DCE-MRI], transcriptomic and pathology.

Initial treatment response showed significant overall decrease in DCE-MRI median K^{trans}, angiogenic factors such *ESM1* and *FLT1*, and proliferation. However, it also revealed great heterogeneity, spanning from downregulation of blood vessel density and central necrosis to continued growth with new vasculature. Crucially, significantly upregulated pathways leading to resistance included glycolysis and pH adaptation, PI3K-Akt and immune checkpoint signaling, for which inhibitors exist, making a strong case to investigate such combinations.

These findings support that anti-angiogenesis trials should incorporate initial enrichment of patients with high K^{trans}, and a range of targeted therapeutic options to meet potential early resistance pathways. Multi-arm adaptive trials are ongoing using molecular markers for targeted agents, but our results suggest this needs to be further modified by much earlier adaptation when using drugs affecting the tumor microenvironment.

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1. Introduction

Angiogenesis is a key process in cancer providing oxygen and nutrients to the growing tumor mass. Key player is the vascular endothelial growth factor [VEGF], targeted by antibodies or small molecule kinase inhibitors. First such agent was bevacizumab [Avastin], monoclonal antibody against VEGF. Clear activity in clinical trials has led to implementation of such therapies as standard of care. However, effects are usually on progression–free rather than overall survival, and often measured in weeks (Carmeliet and Jain, 2000). Whilst benefit has been shown in some cancers, in breast cancer there has been controversy on the role of antiangiogenic therapy (Rossari et al., 2012). Early studies showed improvement of response in metastatic disease with Taxol (Jain, 2014), whilst neoadjuvant studies showed contrasting results (Jain, 2014; Bear et al., 2012; Earl et al., 2015; Sikov et al., 2015; von Minckwitz et al., 2012).

Lack of consensus between clinical trials has questioned the effectiveness of bevacizumab in breast cancer, and the drug is no longer in use in several countries. However, there is large evidence of heterogeneity of response. Therefore we undertook a study in the situation where bevacizumab was approved to understand mechanisms behind this heterogeneity, and clinical implications. Circulating levels of short VEGFA isoforms, neuropilin-1 and VEGF receptor 1 expression in tumours or plasma, and VEGFA genetic variants have been reported as potential biomarkers of bevacizumab response (Lambrechts et al., 2013; Hegde et al., 2013). Changes in DCE-MRI parameters with therapy have been shown, such as volume transfer constant (K^{trans}), complex function of vessel permeability, surface area and tumor blood flow; however, how to use MRI to impact on drug therapy modulation is controversial (O'Connor and Jayson, 2012); reflecting limited insight into molecular

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correlates. A small window study of 21 patients combining DCE-MRI monitoring with limited number of molecular markers, showed reduction of perfusion parameters with increase in tumor apoptosis (Wedam et al., 2006). However, studies to date have been hindered by either combination with chemotherapy, or focus on advance cancers where multiple resistance mechanisms are likely to have developed, or else limited pharmacodynamics assessments probing only some aspects of tumor response.

The present single-agent bevacizumab study, in previously untreated primary breast cancer patients, asks whether DCE-MRI complemented with molecular profiling of response could provide criteria for patient stratification and insight into early actionable pathways of resistance.

2. Methods

2.1. Study Design and Participants

A phase II, non-randomized, open-label study sponsored by Oxford Radcliffe Hospitals NHS Trust (ORH/PID/5575) was given National Research Ethics Services, UK, approval (Oxford REC no. 08/H0604/69). Patient characteristics are shown in Suppl. Table-1 and Consort diagram in Fig. S1. Previously untreated breast cancer patients with either histologically proven locally advanced breast cancer or tumor >3 cm in diameter were included. Bevacizumab was administered as single infusion (15 mg/kg) 2 weeks prior to neoadjuvant chemotherapy. Multiparametric DCE-MRI scans, core biopsies and blood samples were obtained immediately before and 2 weeks after bevacizumab. 47 previously untreated locally advanced primary breast cancer patients were enrolled between July 2008 and November 2010 by the Oxford Radcliffe Hospital NHS Trust ($n = 30$) and Mount Vernon Hospital ($n = 17$). The present study focuses on ductal cancers ($n = 36$); analysis of other subtypes will be presented elsewhere. DCE-MRI data was analysable for 35 patients only.

2.2. Outcomes: DCE-MRI

Treatment response was measured by DCE-MRI. High temporal resolution T1-weighted acquisition was used to images 8–12 central slices of the tumor region. DCE-MRI setup, imaging acquisition details and all image processing and derivation of PK parameters are reported in detail in the Suppl. Methods. Furthermore, all scripts and codes used for the analysis have been submitted to the GitHub public directory: https://github.com/nph/MRI.Side effects reporting followed CTCAEv3.0 (http://ctep.cancer.gov).

2.3. Molecular Profiling

An experienced radiologist obtained the ultrasound guided core biopsies using 14 gauge core biopsy needles with 22 mm throw from the margin of tumours. Affymetrix Human Exon 1.0 ST arrays were used to measure gene expression. To confirm gene expression results, quantitative real-time PCR (qRT-PCR) was performed using same cDNA as used for gene expression array work. Another ultrasound guided core biopsy sample was collected directly on biopsy cassettes immediately immersed in 50mLs of 10% Formalin in standard biopsy pot for immunohistochemistry (IHC) analysis. All samples were processed within 7 days of collection to render formalin-fixed paraffin-embedded (FFPE) blocks (stored at room temperature). Further details on samples handling and storage, and analysis methods are in Suppl. Methods.

2.4. Statistical Analysis

Statistical analysis for DCE-MRI, gene expression profiling and PCR are reported in Suppl. Methods. Changes of immunohistochemistry parameter scores after bevacizumab were analyzed using paired non-parametric method (Wilcoxon Signed Rank Sum, WSRS). Correlation analyses between gene expression scores, MRI parameters, clinical variables, IHC parameters scores were also performed using non-parametric methods (Spearman for continuous, Kruskal-Wallis for categorical variables). GraphPad PRISM v4.0c, Matlab and R v2.13.0 were used.

2.5. Funding

Breast Cancer Research Foundation, Breast Cancer Research Trust, NIHR Oxford Biomedical Research Centre, and Oxford Cancer Research UK Imaging Centre. Roche provided bevacizumab free of charge. EU FP7 p-medicine and Eureca projects supported FMB and RVS.

3. Results

3.1. Highly Heterogeneous Early Response to Bevacizumab

Single-dose bevacizumab administration (15 mg/kg) was well tolerated with no grade 3 toxicity in 36 patients (Table S1). Of six patients presenting erythematous breast mass, four showed reduced erythema (Fig. S2). No changes after treatment could be detected by standard clinical evaluation (Fig. S2); however DCE-MRI analysis revealed significant decrease in median K^{trans} and k_{ep} (WSRS, $P < 0.001$) (Fig. 1a), and Minkowski–Bouligand fractal dimension (WSRS $P = 0.012$, Fig. S3). This suggests general improvement in hierarchical architecture, hence tendency to vasculature normalisation.

DCE-MRI response was heterogeneous (Fig. 1b, S3), with high baseline median K^{trans} tumours showing greatest reduction (Spearman $\rho = -0.92$, $P = 1e-08$, Fig. 1c). Variability in median K^{trans} was also observed within individual tumours (Fig. 1d). Visual assessment of parametric maps confirmed this heterogeneity, revealing patterns from not-significant change to strong median K^{trans} reduction across complete tumor mass (Fig. 1d). As different parameter distributions could result in similar median K^{trans} values, we investigated cumulative K^{trans} over the central tumor region. This new parameter, defined here as *total* K^{trans}, provides an estimate of total target mass presented for modification by bevacizumab, and might help defining heterogeneity (Fig. 2). Total K^{trans} decreased post-bevacizumab (WSRS, $P < 0.0001$) (Fig. 2c), changes were correlated with pre-bevacizumab Total K^{trans} (Fig. 2d), and highly variable (Fig. 2e).

3.2. Angiogenesis is Downregulated Whilst VEGFA Transcript is Induced

Gene-wise paired differential expression analysis revealed 23 significantly (FDR < 0.05) downregulated genes post-bevacizumab (Fig. 3a) (Table S2). High correlation between gene expression arrays and qRT-PCR confirmation was observed (Fig. 3b–c, Figs. S4, S5). Notably, several (6/23) were also downregulated post-bevacizumab in our xenograft models (Masiero et al., 2013) including *GPR56*, inhibitor of VEGF production; *EXO1*, exonuclease promoting cleavage of transcribed immunoglobulin switch regions; *ILF2*, coding for nuclear factor of activated T-cells (NFAT) component.

Top downregulated transcript was endothelial cell specific molecule-1 (*ESM1*), regulated by VEGFA, mediating angiogenesis and invasion. Interestingly, we observed significantly greater *ESM1* downregulation in high grade tumours (Fig. S5). Other downregulated genes confirmed by qRT-PCR were fms-related tyrosine kinase 1 (*FLT1*), encoding a VEGF receptor (*VEGFR*) family member, and delta like ligand 4 (*DLL4*), vascular-specific Notch ligand (Fig. S4). Significant reduction in microvascular area was also observed. Namely, plasmalemma vascular associated protein (PLVAP) showed an average 20% percentage decrease post-treatment (WSRS $P = 0.014$) (Figs. 4, S6).

A 393 significantly upregulated genes were also identified (Fig. 3a, Table S3), including *VEGFA* itself (confirmed by qRT-PCR, Fig. S7) suggesting a negative feedback loop. Interestingly, chemokine receptor *CXCR4*, upregulated by VEGFA, and its ligand chemokine stromal cell-

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