Bevacizumab treatment nullifies Bax up-regulation induced by epirubicin and docetaxel chemotherapy in human breast cancer.

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Chemotherapeutics utilized in breast cancer, including taxane drugs such as docetaxel, exert their function through a variety of mechanisms; one of these is the induction of apoptotic genes¹ like those of the Bcl-2 family². We found through mining published data from the Phase II PROMIX trial³ that treatment with epirubicin and docetaxel chemotherapy resulted in the induction of the Bcl-2 family gene Bax⁴ which functions in the execution of cell death. However, following addition of bevacizumab to the treatment regimen, Bax expression returned to levels even lower than at baseline. This was not the case in glioblastoma patients treated with bevacizumab, demonstrating tissue-specific function of bevacizumab in human cancer⁵. Thus, in patients with breast cancer, bevacizumab antagonizes activation of cell death gene expression induced by epirubicin and docetaxel chemotherapy, demonstrating an undesirable effect of a therapeutic utilized for the treatment of cancer - a disease, a major feature of which is the acquired ability to evade death signaling⁶.

Keywords: breast cancer, bevacizumab, epirubicin, docetaxel, Bax, systems biology of breast cancer, targeted therapeutics in breast cancer.

1 Bevacizumab is a monoclonal antibody utilized for the treatment of breast cancer worldwide,
2 targeted against the vascular endothelial growth factor A, VEGF-A⁷. Bevacizumab was formerly
3 approved by the Food and Drug Administration in conjunction with paclitaxel for the treatment of
4 metastatic breast cancer which had not previously been treated with chemotherapy for HER2-negative
5 breast cancer; this indication was withdrawn on the basis that its full safety and efficacy had not been
6 demonstrated⁸. For the over quarter of a billion women in the European Union, bevacizumab remains
7 approved by the European Medicines Agency for the treatment of metastatic breast cancer⁹. This
8 disparity in rationale for what remains a critical medical issue for women in the United States, Europe,
9 and worldwide is a problem. To understand the transcriptional response to bevacizumab in an unbiased
10 fashion at the transcriptome level, we mined published microarray data from the phase II clinical trial
11 measuring responses to epirubicin, docetaxel and bevacizumab, PROMIX³.

12 **Methods**

13 We used datasets GSE87455³ and GSE150615⁵ for this global differential gene expression
14 analysis in conjunction with GEO2R. GSE87455 was generated using Illumina HumanHT-12 V4.0
15 expression beadchip technology with $n=122$ primary breast tumors at baseline, $n=83$ primary tumors after
16 Cycle 2 of treatment, consisting of only chemotherapy (epirubicin and docetaxel) and $n=70$ following
17 addition of bevacizumab to treatment regimen in patients with breast cancer; analysis was performed
18 using platform GPL10558. GSE150615 was generated using Genentech glioblastoma custom panel
19 GBM_GNE_C1954 technology with tumors from patients with glioblastoma treated with the following
20 therapeutics: $n=173$ primary tumors from patients treated with placebo (control), $n=116$ primary tumors
21 from patients treated with lomustine, $n=166$ primary tumors from patients treated with bevacizumab, and
22 $n=163$ primary tumors from patients treated with lomustine and bevacizumab; analysis was performed
23 using platform GPL28485. The Benjamini and Hochberg method of p -value adjustment was used for
24 ranking of differential expression but raw p -values were used to assess statistical significance of global
25 differential expression. Log-transformation of data was auto-detected, and the NCBI generated category
26 of platform annotation was used. A statistical test was performed to evaluate whether Bax gene
27 expression was significantly between primary breast tumors at baseline, following chemotherapy
28 (epirubicin and docetaxel) only and following both chemotherapy and bevacizumab in humans with
breast cancer using a one way analysis of variance (ANOVA) with Dunnett's multiple comparisons test.
We used PRISM for all statistical analyses of differential gene expression in human breast cancer (Version
8.4.0)(455).

29 **Results**

30 **Bax is differentially expressed in the primary tumors of patients following administration of 31 epirubicin and docetaxel chemotherapy.**

32 We analyzed data from the PROMIX trial³ wherein patients were treated for six cycles; each of
33 the six cycles included treatment with epirubicin and docetaxel; bevacizumab was added to the treatment
34 regimen only after cycle 2. Tumor tissues were sampled at baseline, following administration of 2 cycles
35 of epirubicin and docetaxel (prior to administration of bevacizumab), and after the addition of
36 bevacizumab, at surgery. We took advantage of this unique microarray dataset, with patient tumor tissues
37 available at baseline, as well as before and after bevacizumab to analyze patient tumor tissues
38 comparatively at each time point to determine in an unbiased fashion how gene expression was perturbed
transcriptome-wide, by bevacizumab, by epirubicin and docetaxel, and how these medications interacted
with each other at the level of human gene expression in the breast.

We found that Bax was among the genes most differentially expressed when comparing the total
transcription at baseline and after administration of 2 cycles of docetaxel and epirubicin. When sorting

each of the genes expressed in primary tumors of the breast based on significance of change in expression between tumors at baseline and following 2 cycles of epirubicin and docetaxel, Bax ranked 9 out of 47323 total transcripts (Table 1). Differential expression of Bax in primary tumors of the breast following administration of epirubicin and docetaxel in patients with breast cancer was statistically significant (Table 1; $p=1.25E-14$).

Bax is among the genes most differentially expressed when comparing patient tumors at baseline, after 2 cycles of epirubicin and docetaxel, and after addition of bevacizumab to treatment regimen.

Next we assessed how gene expression changed, globally, following addition of bevacizumab to the chemotherapeutic regimen after cycle 2, following 4 cycles of epirubicin, docetaxel, and bevacizumab, as measured in primary tumors at surgery³. Surprisingly, when comparing tumors at baseline, after 2 cycles of epirubicin and docetaxel, and after addition of bevacizumab, Bax was again among the genes whose expression changed most significantly across each of these three time points (Table 2). When sorting each of the genes based on significance of change in expression between tumors prior to treatment and following addition of bevacizumab to the treatment regimen, Bax ranked 39 out of 47323 total transcripts (Table 2). Differential expression of Bax in primary tumors of the breast following addition of bevacizumab to epirubicin and docetaxel in patients with breast cancer was statistically significant (Table 2; $p=1.51E-21$). Since Bax was again among the genes whose expression changed most significantly, both after 2 cycles of epirubicin and docetaxel and following addition of bevacizumab, we obtained exact mRNA expression values for Bax from each patient tumor at each time point to directly evaluate magnitude and direction of change in Bax expression.

Induction of Bax by epirubicin and docetaxel is lost following addition of bevacizumab to chemotherapeutic regimen.

In primary tumors of the breast, Bax mRNA levels increased to a significant degree following administration of 2 cycles of epirubicin and docetaxel (Figure 1; $p<0.0001$). We calculated a mean fold change of 1.33 ± 0.34 in Bax mRNA levels in patient tumors after 2 cycles of treatment as compared to baseline.

Surprisingly, we found that Bax mRNA returned to baseline levels following administration of bevacizumab after cycle 2. While the change in Bax mRNA expression in patient tumors at baseline and patient tumors after 2 cycles of epirubicin and docetaxel was statistically significant, change in Bax mRNA levels when comparing patient tumors at baseline and following addition of bevacizumab was not statistically significant ($p=0.0757$). We calculated a mean fold change of 1.33 ± 0.34 in Bax mRNA levels in patient tumor biopsies collected after 2 cycles of epirubicin and docetaxel, as compared to baseline (Table 2), but a mean fold change of 0.92 ± 0.18 in Bax mRNA at surgery, once bevacizumab had been added to the epirubicin and docetaxel treatment regiment for 4 cycles.

Tissue-specific functions of bevacizumab in transcriptional modulation of Bax.

To understand more thoroughly the transcriptional response to bevacizumab in human cancer, we studied a multiplexed gene expression dataset generated using tissues from patients with glioblastoma treated with lomustine, bevacizumab, or both, as well tumor tissues from placebo-treated patients⁵. We found that in glioblastoma, Bax was also a differentially expressed gene when comparing tumors based on treatment with nitrosourea chemotherapy lomustine, bevacizumab, bevacizumab and lomustine, or placebo. When sorting each of the genes expressed in glioblastoma tumors based on significance of change in expression between tumors of control patients and the tumors of patients treated with either lomustine, bevacizumab, lomustine and bevacizumab or placebo, Bax ranked 106 out of 786 total transcripts (Table 3), equating to 86.5% differential expression.

Differential expression of Bax in the tumors of glioblastoma patients when comparing tumors based on treatment with either placebo, lomustine, bevacizumab, or lomustine and bevacizumab was statistically significant (Table 3; $p=2.19E-92$).

However, as opposed to taxane chemotherapy in human breast cancer, where Bax expression increased following treatment with docetaxel, Bax expression in the tumors of lomustine-treated glioblastoma patients significantly decreased (Figure 2). Moreover, bevacizumab did not repress or induce the expression of Bax in the primary tumors of patients with glioblastoma (Figure 2; $p=0.8985$), nor did the addition of bevacizumab to lomustine significantly alter the repression of Bax by lomustine (Figure 2; $p=0.9989$). We calculated a mean fold change of 0.901 ± 0.045 in Bax expression in glioblastoma patients treated with lomustine as compared to glioblastoma patients treated with placebo, and 0.901 ± 0.044 in glioblastoma patients treated with lomustine and bevacizumab, but a mean fold change of 0.996 ± 0.052 in Bax expression in glioblastoma patients treated with bevacizumab.

Thus, in human breast cancer, the induction of the apoptotic executioner gene Bax resulting from administration of epirubicin and docetaxel chemotherapy is essentially reversed by addition of bevacizumab to the treatment regimen. This appears to be a tissue-specific function of bevacizumab in human cancer as lomustine chemotherapy represses Bax rather than inducing its expression, and bevacizumab does not alter this repression.

Discussion

Bax has critical functions in the induction of programmed cell death⁹ by heterodimerizing with Bcl-2¹⁰ and inducing pore formation in the mitochondrial membrane, together linking signals transduced at the plasma membrane to changes in mitochondrial permeability that result in apoptosis^{11,12}. Thus, induction of Bax expression by epirubicin and docetaxel should be considered a desirable consequence of chemotherapy administration, and reversal of Bax induction by addition of bevacizumab to epirubicin and docetaxel should be considered detrimental at the organismal level.

These data provide one piece of specific molecular evidence that, in conjunction with United States Food and Drug Administration opinion, challenge the European Medicines Agency medical opinion approving bevacizumab indication for the treatment of breast cancer in conjunction with a taxane chemotherapy (paclitaxel), as the induction of a gene, Bax, with key functions in programmed cell death, is nullified by the addition of bevacizumab in women with breast cancer treated with another taxane chemotherapy, docetaxel. Future data can assess whether any other messenger RNAs for gene products with important functions in control of tumor growth are blunted or lost following addition of bevacizumab to standard chemotherapeutic regimens in women with breast cancer, and care should be taken to more thoroughly understand tissue-specific functions of and transcriptional modulation by complex therapeutics utilized in the treatment of humans with cancer.

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Rank	ID	p-value	t	B	FC	Gene	Gene name
9	ILMN_2321064	1.25E-14	8.3183043	22.67093	0.92 ± 0.18	BAX	BCL2 associated X, apoptosis regulator

Table 1: Bax is among the genes most differentially expressed when comparing primary tumors at baseline and following 2 cycles of epirubicin and docetaxel chemotherapy.

The rank of differential expression, transcript ID, *p*-value with respect to global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression when comparing primary tumors at baseline to primary tumors following administration of chemotherapy only (epirubicin and docetaxel), fold change in Bax expression between primary tumors at baseline and primary tumors following administration of chemotherapy only (epirubicin and docetaxel), gene and gene name are listed in this chart.

Rank	ID	p-value	F	FC	Gene	Gene name
39	ILMN_2321064	1.51E-21	5.74E+01	1.33 ± 0.34	BAX	BCL2 associated X, apoptosis regulator

Table 2: Bax is among the genes most differentially expressed when comparing primary tumors at baseline, following 2 cycles of epirubicin and docetaxel chemotherapy, and following addition of bevacizumab to treatment regimen after cycle 2.

The rank of differential expression, transcript ID, *p*-value with respect to global differential expression, F statistic, fold change in Bax expression between primary tumors at baseline and primary tumors following administration of bevacizumab after cycle 2, gene and gene name are listed in this chart.

Figure 1: Induction of Bax by epirubicin and docetaxel is lost following addition of bevacizumab to chemotherapeutic regimen.

Expression of Bax at the messenger RNA level from the tumors of patients enrolled in the PROMIX trial is graphically depicted here in primary tumors at baseline (left), in primary tumors following administration of 2 cycles of chemotherapy only (epirubicin and docetaxel) ("Chemo", middle), and in primary tumors at surgery, following addition of bevacizumab to treatment regimen ("Chemo + Bev", right). with mean Bax mRNA level marked and the result of a statistical test evaluating significance of difference in Bax expression between tumors at baseline and tumors following epirubicin and docetaxel or bevacizumab and epirubicin and docetaxel, a p-value, listed above. *****: $p < 0.0001$.

Rank	ID	p-value	F	FC	Gene
106	NM_138761.3	2.19E-92	205.4993	L: 0.901 ± 0.045 C: 0.996 ± 0.052 L+C: 0.901± 0.044	Bax

Table 3: Bax is among the genes most differentially expressed in the tumors of patients with glioblastoma following treatment with either placebo, lomustine, bevacizumab, or both lomustine and bevacizumab.

The rank of differential expression, transcript ID, p-value with respect to global differential expression, F statistic, fold change in Bax expression between primary tumors treated with placebo and primary tumors in patients treated with either lomustine (L), bevacizumab (B) or both lomustine and bevacizumab (L+B), and gene symbol are listed in this chart.

Figure 2: Bax is repressed by lomustine chemotherapy in patients with glioblastoma, this is not modulated by bevacizumab, and the addition of bevacizumab to lomustine does not change Bax repression.

Expression of Bax at the messenger RNA level from the tumors of patients with glioblastoma is graphically depicted here in primary tumors treated with placebo (Control), in primary tumors following administration of lomustine chemotherapy (“Lomust”), in primary tumors from patients treated with bevacizumab (“Bev”), and in primary tumors from patients treated with both lomustine and bevacizumab (“Lomust + Bev”), with mean Bax mRNA level marked and the result of statistical tests evaluating significance of difference in Bax expression between primary glioblastomas treated with placebo and glioblastoma treated with either lomustine, bevacizumab, or both, p-values, listed above.

*****: $p < 0.0001$.