JAK2 Expression Is Associated with Tumor-Infiltrating Lymphocytes and Improved Breast Cancer Outcomes: Implications for Evaluating JAK2 Inhibitors


Abstract
Janus kinase-2 (JAK2) supports breast cancer growth, and clinical trials testing JAK2 inhibitors are under way. In addition to the tumor epithelium, JAK2 is also expressed in other tissues including immune cells; whether the JAK2 mRNA levels in breast tumors correlate with outcomes has not been evaluated. Using a case–control design, JAK2 mRNA was measured in 223 archived breast tumors and associations with distant recurrence were evaluated by logistic regression. The frequency of correct pairwise comparisons of patient rankings based on JAK2 levels versus survival outcomes, the concordance index (CI), was evaluated using data from 2,460 patients in three cohorts. In the case–control study, increased JAK2 was associated with a decreasing risk of recurrence (multivariate \(P = 0.003\), \(n = 223\)). Similarly, JAK2 was associated with a protective CI (<0.5) in the public cohorts: NETHERLANDS CI = 0.376, \(n = 295\); METABRIC CI = 0.462, \(n = 1,981\); OSLOVAL CI = 0.452, \(n = 184\). Furthermore, JAK2 was strongly correlated with the favorable prognosis LYM metagene signature for infiltrating T cells (\(r = 0.5; P < 2 \times 10^{-16}; n = 1,981\)) and with severe lymphocyte infiltration (\(P = 0.00003; n = 156\)). Moreover, the JAK1/2 inhibitor ruxolitinib potently inhibited the anti-CD3-dependent production of IFN-\(\gamma\), a marker of the differentiation of Th cells along the tumor-inhibitory Th1 pathway. The potential for JAK2 inhibitors to interfere with the antitumor capacities of T cells should be evaluated. Cancer Immunol Res; 2(4): 301–6. ©2014 AACR.

Introduction
Janus kinase-2 (JAK2) is essential for the signaling of a variety of cytokine receptors, including receptors for erythropoietin during erythropoiesis and for prolactin during mammary differentiation (1–2). JAK2 has emerged as an important target in myeloproliferative disorders, and increasingly, in solid tumors such as breast cancer. Recent studies have implicated JAK2 in interleukin (IL)-6–dependent breast cancer stem cell self-renewal (3), and in both IL-6- and IL-8–dependent growth of triple-negative breast cancers (4). Furthermore, JAK2 signaling has been implicated as a mechanism of escape from other targeted breast cancer therapies (5). Thus, JAK2 inhibitors are being evaluated in patients with breast cancer (6). JAK2 is also expressed in diverse cell types, including immune cells, and whether the overall JAK2 mRNA levels in breast tumors are associated with clinical outcomes has not been evaluated.

Studies of mRNA levels in primary breast tumors have been useful for classifying breast cancers into subtypes that correlate with prognosis and drug responsiveness (7–9), for predicting recurrence (10, 11), and for delineating gene expression signatures that correlate with prognosis (12–15). Here, we evaluated the association between tumor mRNA levels of JAK2 and clinical outcomes in a novel case–control study and in three public cohorts. Outcomes included distant metastatic recurrence in a matched case–control study (\(n = 223\)); recurrence-free survival in the Netherlands Cancer Institute cohort that was used to develop the MammaPrint recurrence risk test (\(n = 295\); ref. 11); overall and disease-specific survival in METABRIC, currently the largest collection of gene expression and copy-number data linked to long-term breast cancer outcomes (\(n = 1,981\); ref. 14); and overall survival in OSLOVAL, a recent cohort that, along with METABRIC, formed the basis of the Sage Bionetworks DREAM breast cancer prognosis challenge (\(n = 184\); refs. 12, 15).

Materials and Methods
Selection of cases and controls
The protocol to use a breast cancer research database for case selection, to access institution-archived leftover tumor tissue, and to undertake molecular biology studies of the tissue was approved by the Institutional Review Boards of the Fred Hutchinson Cancer Research Center (File 6643) and the
Swedish Medical Center (File 6924–10). Patient consent was not required. The breast cancer research database at the Swedish Cancer Institute contains patient, tumor, treatment, and outcomes data collected since 1989 for more than 12,000 patients. The dataset was reduced to women followed for at least 2 years with invasive carcinoma with T1-3 primary tumors and treated by partial mastectomy, sentinel node biopsy or axillary dissection, and adjuvant chemotherapy. Patients with multiple primaries, T4 primaries or distant metastases, and those receiving neoadjuvant chemotherapy, were excluded. Matching variables included extranodal extension of metastasis, lymphovascular invasion, estrogen receptor (ER)/progesterone receptor (PR)/human epidermal growth factor receptor-2 (HER2) status, T-stage, and N-stage. A T–N interaction term allowed for the fact that tumor size is more important for women without positive nodes than for women with positive nodes. Within each matched pair, diagnosis dates of the recurring and nonrecurring patients were no more than 2 years apart. Propensity scoring was used to match 112 cases of distant recurrence following surgery to 112 nonrecurring controls using the ‘Optmatch’ package (16) and R (17).

Quantitative Reverse Transcriptase PCR
RNA was extracted from 4 × 10 μm sections using the Absolutely RNA FFPE System (Stratagene). The amount of tumor versus normal tissue in each section was greater than 50% for 84% of samples and greater than 90% for 47% of samples as determined by pathologists’ inspection of hematoxylin and eosin–stained slides. cDNA was synthesized using random hexamers and SuperScript III (Invitrogen) and was preamplified for 14 cycles using the Taqman preamplification system (Applied Biosystems). All probes bound exon junctions to prevent genomic DNA amplification (Supplementary Table S1). Diluted cDNA was used to seed triplicate real-time PCR reactions for each Taqman assay using standard cycling conditions. Cycle threshold (Ct) values were determined using Sequence Detection Software (Applied Biosystems). Relative quantification was calculated as 2^(-ΔCt), where ΔCt values were calculated by subtracting the indicated control gene mean Ct value from the target gene mean Ct value.

JAK2 mRNA levels and distant recurrence in the case–control study
Associations between JAK2 mRNA and the likelihood of recurrence were evaluated by logistic regression. In the continuous model, coefficients were calculated as estimates of the change in the log of the odds that an individual experienced a recurrence for every 2-fold increase in JAK2 levels, in which a negative coefficient indicates that increasing JAK2 levels are associated with a decreasing likelihood of recurrence. Because the participants had one (n = 184), two (n = 26), or three tumor specimens (n = 14), generalized estimating equations were used to account for varying numbers of tissues per individual (‘Geepack’ package ref. 18). Regressions were also performed using only primary tumors. For this, among the 26 individuals with both a primary and node specimen, only the primary tissue was included, and data from 31 individuals with only a node specimen were excluded. In the dichotomous model, coefficients were calculated with above-median versus below-median JAK2 levels as a predictor of recurrence, in which multiple specimens were averaged to one value per individual. Multivariate analysis adjusted for clinical factors with which JAK2 expression was significantly correlated.

JAK2 mRNA levels and survival outcomes in the public cohorts
The inclusion criteria, clinical characteristics, and follow-up of the NETHERLANDS, METABRIC, and OSLOVAL cohorts were described (11, 12, 14). Data are available via Sage Bionetworks (www.synapse.org) under the following identifiers: doi:10.7303/syn6517.1, doi:10.7303/syn1688369, and doi:10.7303/syn1688370. Concordance index (CI) values were determined as described (12, 19).

Results
Case–control study of JAK2 mRNA levels and distant recurrence
We previously optimized methods for measuring JAK2 mRNA in formalin-fixed, paraffin-embedded tumors by quantitative reverse transcriptase PCR (qRT-PCR) despite the degradation that characterizes RNA extracted from these samples (20). We applied this approach to tumor specimens from 112 women who underwent surgery for breast cancer and subsequently experienced a distant metastatic recurrence and 112 women who did not. With the exception of a borderline significant increase in the number of ER+ tumors among recurrences, there were no significant differences in clinical characteristics between cases and controls (Supplementary Table S2). Of note, although ER was a matching variable, it was not the only variable, which accounts for the residual effect of this strong prognostic factor even after propensity score matching. Sufficient RNA was available in 223 tumor specimens for JAK2 mRNA determinations. The validity of our mRNA measurements was confirmed by (i) the strong correlation in values obtained using probes for both JAK2 exon8/9 and exon23/24; (ii) the reproducible mRNA levels across three separate tumor specimens for 14 tumors for which this comparison was possible; and (iii) the strong concordance in mRNA levels of ESR1, PGR, ERBB2, and the corresponding clinical immunohistochemistry results (Supplementary Fig. S1).

JAK2 mRNA levels were significantly higher in tumors from women who experienced no distant recurrence compared with those who experienced a distant recurrence (Fig. 1A). This association was significant for both JAK2 exon8/9 and exon23/24 probes in logistic regression when JAK2 mRNA was treated as a continuous or dichotomous variable (Supplementary Table S3). Furthermore, with the exception of the JAK2 exon8/9 probe in the dichotomous model, significance was maintained in multivariate analysis. The association between increasing JAK2 mRNA and decreasing distant recurrence was also significant when analysis was restricted to primary tumors. A receiver operator curve revealed that the association between higher JAK2 mRNA and reduced recurrence was maximal when tumor samples with the top 40% to 50% of JAK2 expression level were defined as high JAK2 (Fig. 1B).
**JAK2 mRNA levels and survival outcomes in the public cohorts**

Next, we evaluated the association between JAK2 mRNA and outcomes in the NETHERLANDS, METABRIC, and OSLOVAL cohorts. We used the CI (19), which provides a convenient measure of the strength and direction of an association between a single gene and outcomes, to score submissions in the Sage Bionetworks DREAM breast cancer prognosis challenge (12). The CI is the relative frequency of correct pairwise comparisons of patient rankings based on gene expression levels versus survival outcomes. A CI > 0.5 indicates that higher expression is associated with shorter survival, whereas a value of <0.5 indicates that higher expression is associated with longer survival. For example, using disease-specific survival data in METABRIC, the single-gene mRNA with the poorest prognosis was previously found to be CDCAS with a CI of 0.651, indicating that if 2 patients were randomly selected, the patient with the higher CDCAS level will have shorter survival 65.1% of the time (12). Conversely, the single most protective gene was FGD3 with a CI of 0.352, indicating that if 2 patients were randomly selected, the patient with the higher FGD3 level will have the longer survival 64.8% (100%–35.2%) of the time.

JAK2 mRNA exhibited a protective CI in all three datasets (Fig. 2). The strongest effect was observed in the NETHERLANDS cohort, in which the CI of 0.376 indicates that if 2 patients were randomly selected, the patient with the higher tumor JAK2 mRNA level would have the longer recurrence-free survival 62.4% (100%–37.6%) of the time. Similarly, JAK2 mRNA was consistently protective, albeit to a lesser extent, in the METABRIC and OSLOVAL cohorts. Because METABRIC provided a sufficient sample size, we also evaluated the CI for JAK2 mRNA for each indicated cohort. For comparison, the CIs for the least and most protective single genes in METABRIC (CDCAS and FGD3) are shown.
mRNA in ER− and ER+/PR−/HER2− (triple-negative) subtypes; JAK2 mRNA was even more protective for both overall and disease-specific survival in these subtypes.

**JAK2 mRNA and protein levels**

To explore the mechanism by which JAK2 mRNA is associated with favorable prognosis, we investigated the relationship between JAK2 mRNA and protein levels. The specificity of a total JAK2 antibody was validated by the strong correlation between JAK2 mRNA and protein levels (as measured by Western blotting) in a panel of 18 human breast cancer cell lines and by the ability to discriminate between JAK2-deficient γ2A cells (21) and JAK2-transfected γ2A cells in immunohistochemistry (Supplementary Figs. S2 and S3). We then measured 10 tumors from the case–control study randomly selected from the highest quartile of JAK2 mRNA expression and 10 tumors from the lowest quartile. Immunohistochemical staining ranged from absent (0) to robust (3+) and was prominent in the tumor epithelial cells. However, JAK2 antibody staining levels in the tumor epithelial cells were not correlated with overall tumor JAK2 mRNA levels (r = −0.17, n = 20; Supplementary Table S4). This finding suggests that the association between higher JAK2 mRNA levels and favorable outcomes in breast cancer may not be a result of JAK2 protein function in breast tumor epithelial cells, and that other cell types in the primary tumors that are associated with prognosis contribute to overall JAK2 mRNA levels.

**JAK2 mRNA levels and tumor-infiltrating T cells**

JAK2 is expressed in immune cells, and tumor-infiltrating lymphocytes, especially T cells, have been associated with favorable breast cancer prognosis (14, 22). We, therefore, tested whether breast tumor JAK2 mRNA levels correlate with the T-cell transcript-enriched LYM metagene signature. The LYM metagene is associated with favorable prognosis in breast cancer, in particular in ER− breast cancer and even more so in the absence of multiple positive lymph nodes, and recently formed part of the winning prognostic model in the Sage Bionetworks DREAM breast cancer prognosis challenge (12, 15). The LYM metagene was recently defined with increased accuracy, following mining from data sets from multiple cancer types available from The Cancer Genome Atlas (23). Indeed, there was a highly significant correlation between JAK2 mRNA levels and the LYM metagene in tumor samples from METABRIC (Fig. 3A). In contrast, the LYM metagene had an inverse correlation with the breast epithelial-associated transcript ESR1 (Fig. 3B). Furthermore, JAK2 mRNA levels were correlated strongly with levels of infiltrating lymphocytes as determined by pathologic assessment in a subset of 156 tumors for which these data were available (Fig. 4). These tumor samples belonged to METABRIC integrative cluster 4, which was previously associated with favorable prognosis and a strong adaptive immune response signature (14). Finally, consistent with a functional role for JAK2 in supporting cytokine receptor signaling during T-cell activation, we found that the JAK1/2 inhibitor ruxolitinib markedly inhibited the anti-CD3-dependent production of IFN-γ, a marker of the differentiation of Th cells along the tumor-inhibitory Th1 pathway (Supplementary Fig. S4; 24). These results suggest that the consistently protective effect of JAK2 mRNA is related, at least in part, to the levels of infiltrating T cells.

**Discussion**

To our knowledge, this is the first time that a consistent association between increasing JAK2 mRNA levels and improved breast cancer outcomes has been demonstrated. This association was strongest in the case–control study that matched for variables associated with recurrence. Although the influence of JAK2 mRNA on survival outcomes in the unmatched public cohorts was predictably not as strong, the remarkably consistent association between higher JAK2 mRNA and favorable survival is unexpected because JAK2 proteins collaborate with a variety of cytokine receptors that were shown to promote breast cancer growth (3, 4). The association of JAK2 mRNA with favorable prognosis may reflect a lack of concordance between the levels of JAK2 mRNA and total JAK2 protein and the active phospho-JAK2 in breast tumor epithelial
The association between JAK2 mRNA and favorable prognosis also likely reflects the presence of additional JAK2-expressing cell types in the tumor specimens. Consistent with this finding, our previous laser capture microdissection studies demonstrated that JAK2 mRNA was expressed in both breast tumor epithelial and nonepithelial fractions, but was 8.3 (1.5–44.6)-fold higher in nonepithelial fractions (25). Indeed, we observed a strong correlation between JAK2 mRNA and levels of tumor-infiltrating lymphocytes and the favorable prognosis LYM metagene signature. Our finding that a single gene correlates with a larger biomolecular metagene that is associated with prognosis is reminiscent of the frequent association between single genes and the PCNA and CIN metagene signatures for proliferation and chromosomal instability (12).

In addition to the present demonstration that the JAK1/2 inhibitor ruxolitinib inhibits CD3-dependent Th1 differentiation, support for a role of JAK2 in T cells is provided by studies demonstrating that JAK2 is involved in the signaling of IL-12 and IFN-γ, key regulators of the tumor-inhibitory Th1 response (24, 26, 27). Furthermore, JAK inhibitors have been shown to impair production of these Th1 cytokines (28) and to inhibit IFN-γ–dependent T-cell trafficking in murine preclinical studies (29). Determining how specific inhibition of the individual JAK family members influences the repertoire and antitumor activities of tumor-infiltrating T cells represents an important area for future investigation. Such studies will provide insights into whether the benefits of inhibiting JAK2 in breast tumor cells outweigh the potential risks of inhibiting tumor-infiltrating T cells.

Disclosure of Potential Conflicts of Interests
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C.P. Miller, J.D. Thorpe, J.D. Beatty, N.D. Urban, C.A. Blau
Development of methodology: C.P. Miller, J.D. Beatty, N.D. Urban
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.P. Miller, A.N. Kortum, J.D. Beatty, C.A. Blau
Writing, review, and/or revision of the manuscript: C.P. Miller, J.D. Thorpe, A.N. Kortum, C.M. Coy, D. Anastassiou, J.D. Beatty, N.D. Urban, C.A. Blau
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.D. Thorpe, C.M. Coy, J.D. Beatty
Study supervision: C.P. Miller, J.D. Beatty, N.D. Urban, C.A. Blau

Acknowledgments
The authors thank Hailing Lu, Brigham Mechem, and Michiel Schummer for insightful discussions, which improved the interpretation of the results; Mary Atwood for providing patient clinical characteristics; Desérée Iraire and Kathy O’Briant for acquisition of pathology specimens; Ronald Tickman and Sean Thornton for tumor-block selection; Brian Johnson and Peggy Porter for immunohistochemistry and interpretation; Jeanne Lee for assistance in harvesting tumor tissue; Dan Herendeen, Ekram Gad, and Benjamin Curtis for providing murine splenocytes; Hsuan-Ni Lee, Nancy Zhang, and Gahlen Chen for assistance in analyzing data; and Erica Jolin for assistance with human subjects.

Grant Support
This work was supported by grants from the National Cancer Institute (1R01CA135357 to C.A. Blau), Avon (P50CA083636-S2 to N.D. Urban), and the American Cancer Society (117662-MRSG-09-268-01-CCE to C.P. Miller). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 23, 2013; revised December 17, 2013; accepted January 2, 2014; published OnlineFirst January 15, 2014.

References


Cancer Immunology Research

**JAK2 Expression Is Associated with Tumor-Infiltrating Lymphocytes and Improved Breast Cancer Outcomes: Implications for Evaluating JAK2 Inhibitors**


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-13-0189

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2014/01/15/2326-6066.CIR-13-0189.DC1

Cited articles
This article cites 26 articles, 10 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/2/4/301.full.html#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at
pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at
permissions@aacr.org.