Potential Refinement of Recurrence Score by pSTAT3 Status

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2. Methods

2.1. Tissue Analysis:

Formalin fixed, paraffin-embedded invasive breast cancer tissues from hormone receptor (HR) positive, HER2-negative, early breast carcinoma patients treated between the years 1996–2013 were available from the Department of Pathology.

Tissue cores with a diameter of 0.6 mm were taken with a manual tissue array maker MTA-1 (Beecher Instruments) arrayed in duplicates or triplicates on a recipient paraffin block and placed on charged poly-lysine-coated slides [12].

The immunodetection and scoring of pSTAT3 was carried out as previously described, and the staining was determined according to the Remmele score (0–6, intensity + percentage, intensity (0, negative; 1, weak; 2, moderate; 3, strong) + percentage of the stained tumor cells (0, <10%; 1, 10–25%; 2, 25–50% and 3, 50–100%)) [12].

Nuclear Phospho-STAT3 staining was determined separately for each specimen (Figure 1).

Positive pSTAT3 IHC was defined as score \( \geq 2 \).

2.2. Clinical Data

Demographic, clinical, and recurrence data were obtained from electronic medical records. The use of tumor specimens and data in this research was approved by the Medical Ethics Committee of the Hadassah Medical Center, and each patient gave written informed consent.

2.3. Statistical Analysis

The statistical analysis was generated using SPSS software version 27. Chi-square test for categorical data and unpaired Student’s t-test for continuous variables were used. Estimated DFS and overall survival (OS) rates according to the pSTAT3 score subgroups were calculated by the product limit method (Kaplan–Meier) for all patients and were stratified by age at diagnosis; 95% confidence intervals (CIs) were calculated from the model. Multivariate models were computed with Cox proportional-hazards regression. \( p \) values \( \leq 0.05 \) were considered statistically significant.

3. Results

The study population comprised of 449 women with interpretable pSTAT3 IHC. The median age at diagnosis was 52. A comparison between pSTAT3 positive versus pSTAT3 negative tumors did not demonstrate a significant relationship between pSTAT3 positivity and age, histological grade, tumor size, histological type, and involved lymph node number (\( p > 0.05 \)). Furthermore, we have analyzed the relation between pSTAT3 status and survival.
in 436 patients with known outcome. The median follow up was 105 months (censored at 10 years of follow up). In pSTAT3-positive tumors, the 10-year overall survival was non-significantly greater than pSTAT3 negative tumors (78.1% vs. 69.4%, \( p = 0.17 \)). A multivariate analysis adjusted for stage, age, and grade did not demonstrate an association of pSTAT3 with overall survival (hazard ratio for death, 0.72 (95% CI, 0.41–1.28); \( p = 0.26 \)).

To obtain additional insight into the role of pSTAT3 in prognostication, we have analyzed a subset of 75 patients with known RS results (Table 1). Of them, 16 patients (21.3 %) had breast cancer recurrence, and one patient suffered from metastatic second primary tumor (lung cancer). The median age on primary diagnosis was 70.15. The median follow-up time was 114 months.

Table 1. Patient characteristics according to pSTAT3 Score.

| Sub-Variable               | pSTAT3 Score, Negative (<2)/Positive (≥2) | \( n = 75 \) |
|----------------------------|------------------------------------------|--------------|
|                            | Negative (\( n = 49 \))                  | Positive (\( n = 26 \)) | \( p \) Value |
| Age at diagnosis           |                                          |              |              |
| Over 50                    | 38 (77.6%)                               | 18 (69.2%)   | 0.43         |
| Under 50                   | 11 (22.4%)                               | 8 (30.8%)    |              |
| Tumor type                 |                                          |              | 0.966        |
| Ductal                     | 35 (71.4%)                               | 17 (65.4%)   |              |
| Lobular                    | 5 (10.2%)                                | 3 (11.5%)    |              |
| Mucinous                   | 1 (2.0%)                                 | 1 (3.8%)     |              |
| Both Ductal and Lobular    | 8 (16.3%)                                | 5 (19.2%)    |              |
| Lymph Nodes                |                                          |              | 0.951        |
| No                         | 38 (77.6%)                               | 20 (76.9%)   |              |
| Yes                        | 11 (22.4%)                               | 6 (23.1%)    |              |
| Chemotherapy               |                                          |              | 0.357        |
| No                         | 33 (67.3%)                               | 20 (76.9%)   |              |
| Yes                        | 16 (32.7%)                               | 6 (23.1%)    |              |
| Hormone therapy            |                                          |              | 0.706        |
| No                         | 6 (12.2%)                                | 2 (7.7%)     |              |
| Yes                        | 43 (87.8%)                               | 24 (92.3%)   |              |
| RS score                   |                                          |              | 0.15         |
| ≤26                        | 41 (83.7%)                               | 25 (96.2%)   |              |
| >26                        | 8 (10.3%)                                | 1 (3.8%)     |              |
| Recurrence                 |                                          |              | 0.007        |
| No                         | 34 (69.4%)                               | 25 (96.2%)   |              |
| Yes                        | 15 (30.6%)                               | 1 (3.8%)     |              |
| Age                        | Median (range)                           | 58.01 ± 12.07|              |
|                            | Mean ± SD                               | 57.88 ± 10.68|              |

Nine patients (12%) had a high RS of 26 or more. Additionally, 36% of patients had positive pSTAT3 scores (≥ 2). It is noteworthy that patients in low and high RS groups (≤25, 26 and more, respectively) had similar characteristics in regard with age, tumor grade, histological type, tumor size, and lymph node number.

When patients were divided according to their pSTAT3 IHC scores (Figure 2a), we observed that positive scores were significantly associated with a reduced risk of recurrence (\( p = 0.005 \)); out of the 16 patients that had disease recurrence, only one was positive for pSTAT3.

Additionally, patients’ allocation with negative pSTAT3 scores (<2) to high or low RS (with a cutoff of 26) showed that both groups have similar percentage of recurrence.

On the other hand, when patient allocation started according to RS scores (Figure 2b) and then we dichotomized according to positive or negative pSTAT3, the low RS/high pSTAT3 group had a significantly better prognosis (\( p = 0.007 \)).

DFS analysis according to the pSTAT3 score did not show a statistically significant difference between the ‘negative’ groups and the ‘positive’ group (Figure 3a, \( p = 0.109 \)). Yet, DFS analysis according to the pSTAT3 score showed a statistically significant difference between the ‘negative’ groups and the ‘positive’ group when adjusted by age at diagnosis (Figure 3b, \( p = 0.03 \)).
Figure 2. (a) Patients divided according to their pSTAT3 IHC scores; (b) patient allocation started according to RS scores.

Figure 3. Kaplan–Meier analysis for DFS (a) according to the pSTAT3; (b) according to the pSTAT3 and adjusted by age at diagnosis.

Multivariate analysis of the clinical and pathological data for DFS was performed (Table 2). We found that several clinical and pathological characteristics were associated with worse DFS, including age under 50 at diagnosis ($p = 0.039$) and Lobular type ($p = 0.024$).

Table 2. Multivariate analysis for DFS.

| Multivariate Analysis of the Clinical and Pathological Data for DFS | Adjusted HR | 95.0% CI for HR | $p$ Value |
|---------------------------------------------------------------|-------------|----------------|-----------|
| RS score, Neg/Pos (>26)                                       | 0.548       | 0.089–3.394    | 0.518     |
| Lymph Nodes                                                   | 2.709       | 0.405–18.119   | 0.304     |
| Type: Ductal/Lobular/Mucinous/Ductal & Lobular                | 14.367      | 1.261–163.673  | 0.032     |
| Type: Ductal vs. Lobular                                      | 39.831      | 2.747–577.569  | 0.007     |
| Type: Ductal vs. Mucinous                                     | 3.839       | 0.893–16.502   | 0.071     |
| Type: Ductal vs. Ductal and Lobular                           | 0.556       | 0.118–2.623    | 0.458     |
| Hormone therapy                                               | 1.946       | 0.302–12.532   | 0.483     |
| Tumor Size (cm)                                               | 0.981       | 0.438–2.198    | 0.963     |
| pSTAT3 Score, Negative/Positive (≥2)                          | 5.462       | 0.584–51.121   | 0.137     |
| Age at diagnosis under 50                                      | 5.778       | 1.092–30.560   | 0.039     |

The hazard ratio of RS score (<26/≥26) is 0.548 ($p = 0.518$), and the hazard ratio of pSTAT3 score is 5.462 ($p = 0.137$).
4. Discussion

In our preliminary analysis, we have partially recapitulated the known association between positive pSTAT3 to better prognosis and found that pSTAT3 scores might potentially add valuable prognostic data to RS in assessing patients’ risk of early, HR-positive breast cancer recurrence.

As STAT3 functions as a key player in numerous pro-tumorigenic processes, one may expect that its activation will be associated with poor prognosis. However, the reality is far more complex, as shown in previous publications on pSTAT3 as being both a poor and improved prognosis biomarker. Interestingly, this phenomenon was also shown in other tumor types, such as STAT3 contradictory roles in non-small lung cancer [17].

STAT3, as was mentioned, has been shown to play key roles in the cell’s cycle and is mostly considered growth promoting and an antiapoptotic factor. In normal conditions, STAT3 activation is terminated by suppressors of cytokine signaling proteins.

However, cancer cells exhibit constitutively activated STAT3, which is attributable to upregulated tyrosine kinases and the deregulation of its negative regulation by suppressors of cytokine signaling proteins.

STAT3 activation and its target genes in the cell stimulate growth, angiogenesis, and cell motility and inhibit apoptosis. In addition to cell survival and proliferation, STATs regulate several other biological processes that may contribute to cancer. STAT3 has been shown to promote angiogenesis and may contribute to cancer by allowing tumors to evade detection by the host immune system.

One of the important cytokines in this mechanism is interleukin-6 (IL6).

IL6, binding to its receptor complex IL6R/gp130, activates downstream Janus kinases, which subsequently activate the signal transducer and activator of STAT3 through phosphorylation.

Interestingly, IL6/STAT3 signaling has been shown to play a role in tumor progression by inducing epithelial to mesenchymal transition and angiogenesis. In breast cancer, STAT3 is most often activated by IL6 [18].

In fact, many breast cancer cell lines produce IL6, which activates STAT3 by signaling through the IL-6 receptor and Jak kinases [18,19].

One would expect that tumors that express constitutive phospho-STAT3 expression would be associated with poorer prognosis, but our results show otherwise.

In order to resolve this seemingly troublesome inconsistency, we theorized that in clinic, pSTAT3 has a predictive role for adjuvant chemotherapy benefit in breast cancer patients. Dien et al. found a trend, although the results were not statistically significant for longer survival in patients with increased STAT3 activation, and hypothesized that pSTAT3 regulates Tissue Inhibitor of Metalloproteinase-1 (TIMP1), which makes breast cancer cells less invasive [20].

Furthermore, other studies showed that STAT3 is a tumor suppressor protein with a role in breast tissue cellular differentiation and apoptosis [21,22]. Couto et al. found that siRNA knockdown of STAT3 resulted in significantly increased tumor growth in thyroid cancer cell lines. This study also found a correlation between the STAT3 knockdown and increased glucose uptake and the production of lactate in the cell lines. Those finding suggest that one of the mechanisms of the tumorigenesis inhibition character of STAT3 is mediated by inhibiting aerobic glycolysis [23].

Finally, Lee et al. found that STAT3 mediates tumor suppressor effects by binding to GSK3β, which, in turn, promotes the phosphorylation and the degradation of Snail, a critical regulator of the EMT and cancer metastasis [24].

Evidence that STAT3 plays a role in cellular differentiation and apoptosis, functions as a tumor suppressor, and regulates the in-cell process that decreases cancer cell invasiveness may be consistent with better outcomes in breast cancer patients with high pSTAT3.

A possible explanation for our findings can be drawn from a recent computational analysis of pSTAT3 phenotype [5]. In that work, researchers have shown that patients in the luminal A population were much more likely to possess a pSTAT3 high phenotype, whereas those in the luminal B population were much more likely to have a pSTAT3 low phenotype.
5. Conclusions

In modern oncology, clinicians’ decisions on adjuvant chemotherapy are complex and based on a variety of factors: clinical, pathologic, and molecular. Yet, much room for improvement and personalization exists. In the present trial, we correlate pSTAT3 to RS and identify another and novel potential population (low RS\low pSTAT3) that might benefit from chemotherapy despite low RS (<26). Furthermore, pSTAT3 immunohistochemistry as a simple stand-alone test may possibly also provide prognostic data [4,5,25–27].

Taken together, our observations imply that a double-low score (low RS\low pSTAT3) is a marker of unfavorable outcome in HR-positive breast cancer, mainly in patients under the age of 50 on primary diagnosis. If confirmed in a larger-designed prospective randomized trial, our findings would indicate that pSTAT3 testing could be used as an adjunct biomarker to direct rational adjuvant treatment in luminal breast cancer patients.

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