Proliferation and apoptosis in PhIP-induced rat mammary gland carcinomas with elevated phosphotyrosine-STAT5a

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Abstract In the present study we addressed whether proliferation and apoptosis in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced rat mammary gland carcinomas were different between carcinomas with high and low expression of phosphotyrosine (pY)-STAT5a. We determined that carcinomas with high pY-STAT5a were more proliferative (MIB5 immunostaining) and had a higher expression of cyclin D1 and estrogen receptor α. Furthermore, carcinomas with elevated pY-STAT5a demonstrated lower apoptosis as measured by the TUNEL assay and the Bcl-2 to Bax ratio, and showed increased expression of the long and short isoforms of the prolactin receptor. The results of this study are consistent with the notion that activated STAT5a may provide a growth advantage in some types of mammary gland cancers.

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Keywords: 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP); Rat mammary gland; Breast cancer; STAT5a; Proliferation; Apoptosis

1. Introduction

Signal transducer and activator of transcription 5 (STAT5) is a transcription factor involved in lobuloalveolar development, differentiation and milk protein production in the breast that has been implicated in breast cancer pathogenesis [1–4]. Of the two STAT5 isoforms, STAT5a plays a more prominent role in normal mammary gland development and differentiation [2,5,6]. STAT5a is involved in cell survival in the normal mammary gland and has been shown to regulate proliferation of mammary alveolar epithelial cells [6,7].

Chemically-induced mammary gland cancer in the rat is a longstanding model of human breast cancer. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a heterocyclic amine carcinogen found in cooked meat, has been implicated as a dietary etiological factor for human breast cancer [8,9]. We previously observed by microarray analysis that PhIP-induced rat mammary gland carcinomas show increased expression of STAT5a [10]. Through immunohistochemical analysis, nuclear-localization of STAT5a (putative phosphorylated and activated STAT5a) correlated with carcinomas that were more proliferative and poorly differentiated [11]. To further assess the role of activated STAT5a in breast cancer, the current study measured phosphotyrosine (pY)-STAT5a, proliferation and apoptosis across a collection of PhIP-induced rat mammary gland carcinomas. In addition, we examined the expression of the prolactin receptor (PRLR), known to affect phosphotyrosine-STAT5a (pY-STAT5a) levels through the JAK-STAT5 pathway [12].

2. Materials and methods

2.1. Animals and carcinogen treatment

Mammary carcinomas were archival samples from female Sprague–Dawley rats treated with PhIP-HCl and placed on a high fat diet. Carcinomas and age-matched non-treated normal mammary glands had been stored at −80 °C or formalin-fixed and paraffin-embedded [11,13].

2.2. Immunohistochemical analysis and the TUNEL assay

Immunohistochemistry was performed using the LSAB2 system (DakoCytomation, Carpinteria, CA) according to Shan et al. [11] with minor modifications. The slides of 16 carcinomas classified by immunoprecipitation analysis to have high or low pY-STAT5a levels, and four normal glands were incubated in duplicate with Ki-67 (1:50; Clone MIB5; M7248) or labeled for apoptotic cells with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA), according to the company’s protocol. Quantitation of MIB5 stained cells and apoptotic cells were performed in high-power (40× objective) fields for an approximate number of 1000 cells for each specimen. Measurements were performed in two sections per specimen and an average was obtained.

2.3. Immunoprecipitation analysis and immunoblotting

Mammary gland tissue was homogenized as described in Papaconstantinou et al. [14]. For determination of phospho-STAT5a by immunoprecipitation, 500 µg of total protein lysate from 21 carcinoma samples and four normal mammary gland controls were pre-cleaned with 0.5 µg of rabbit IgG and precipitated with 20 µl of Protein A/G agarose (Santa Cruz). For STAT5a precipitation, 5 µl of STAT5a antibody (Santa Cruz, N-20) were added to the supernatant and an average was obtained.

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Abbreviations: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; PRLR, prolactin receptor; ERα, estrogen receptor α; pY-STAT5a, phosphotyrosine-STAT5a

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antibodies derived against cyclin D1 (H-295), bcl-2 (N–19) and bax (N–20) from Santa Cruz, estrogen receptor α (ERα) (DAKO), prolactin receptor (R&D clone 218616) followed by HRP-conjugated secondary antibodies. Beta-actin (Sigma clone AC–15) was used as an internal loading control.

3. Results and discussion

3.1. Tumor classification

Rat mammary gland carcinomas induced by PhIP were classified into two groups based on their phosphotyrosine-STAT5a (pY-STAT5a) levels as determined by immunoprecipitation analysis (Fig. 1A). One group consisted of 10 carcinomas that had pY-STAT5a protein levels similar to the normal mammary gland. The other group consisted of 11 carcinomas showing at least a 3-fold higher expression of pY-STAT5a (Fig. 1B).

Fig. 1. Immunoprecipitation analyses for classifying PhIP-induced rat mammary carcinomas into high and low pY-STAT5a groups. (A) The figure shows a representative immunoblot of two normal (non-treated) age-matched rat mammary glands, four carcinomas classified in the low pY-STAT5a group and five carcinomas classified in the high pY-STAT5a group. The blot was stripped and reprobed to measure total STAT5a from the reprobed blot. The normalized ratios obtained were used to compare the normalized ratio of the bands was normalized to the density of the respective actin band. The normalized ratios obtained were used to compare the normalized ratio of the bands was normalized to the density of the respective actin band. The normalized ratios obtained were used to compare the normalized ratio of the bands was normalized to the density of the respective actin band. The normalized ratios obtained were used to compare the normalized ratio of the bands was normalized to the density of the respective actin band.

3.2. MIB5 immunostaining and the TUNEL assay

MIB5 antigen staining was used as an indicator of proliferating cells in rat mammary gland carcinomas. The percentage of cells stained for MIB5 was 5.4 times higher in carcinomas with high pY-STAT5a compared to carcinomas with low pY-STAT5a (Fig. 2; P < 0.05). This finding is consistent with our previous observation that carcinomas with nuclear STAT5a have a higher PCNA nuclear labeling index [11]. In both carcinoma groups the percentage of proliferative cells was higher than in normal mammary gland controls (P < 0.05). As assessed by the TUNEL assay, the percentage of apoptotic cells in carcinomas with high pY-STAT5a was 2.8 times lower than in carcinomas with low pY-STAT5a (Fig. 2; P < 0.05). Both carcinoma groups examined showed higher percentage of apoptotic cells, compared to normal mammary glands. The overall growth of the carcinomas as estimated by the proliferation to apoptosis ratio was 10 times higher in tumors with high pY-STAT5a (P < 0.05).

3.3. Immunoblotting

Cyclin D1, a cell-cycle protein, was 3-times higher in carcinomas with high levels of pY-STAT5a (Fig. 3, P < 0.0.5). Previous studies have reported that STAT5a, through the Jak2/STAT pathway, regulates the expression of cyclin D1 [6,15], and that dominant negative STAT5 mutants are repressors for cyclin D1 promoter activity [16,17]. Although there are potentially several mechanisms to account for increased cyclin D1 expression, the results of the current study raise the possibility that transcriptional activation of STAT5a contributes to elevated cyclin D1 in this tumor model.

Estrogen receptor (ER) α, a transcription factor that correlates with proliferative mammary carcinomas and tends to colocalize with cyclin D1 in our rat model [18], was 2.5 times more abundant in carcinomas with high compared to low pY-STAT5a (Fig. 3, P < 0.05). There was no difference between high pY-STAT5a carcinomas and normal mammary gland. The possibility that STAT5a transcriptionally regulates ERα expression in our tumor model requires further study. Studies in the rat corpus luteum suggested that prolactin can transcriptionally regulate the ERs through STAT5a [19].

The ratio of the protein levels of Bcl-2, an anti-apoptotic, and Bax, a pro-apoptotic protein was used to estimate apoptosis in the carcinomas. In accordance with the TUNEL assay data, carcinomas with high pY-STAT5a levels had more than a 3-fold higher ratio of Bcl-2/Bax (Fig. 3). STAT5 is a survival factor in the mammary gland [7,20], and in hematopoietic malignancies [21–24]. Interestingly, dominant-negative forms of STAT5 induce apoptosis in ER-positive breast cancer cells [25].

In the mammary gland the PRLR signaling through the Jak2-STAT5 pathway, resulting in STAT5a phosphorylation, is essential for lobulovascular growth and the activation of genes involved in differentiation and lactogenesis [6,26–28]. We therefore examined the protein levels of the short and long isoforms of the rat PRLR (Fig. 4). Prolactin receptor long was two times higher in carcinomas with high compared to low pY-STAT5a (P < 0.05). PRLR short was 2.4 times higher in carcinomas with high compared to low pY-STAT5a and was 3.3 times higher than in normal glands (P < 0.05). We previously observed an increase in prolactin receptor mRNA in PhIP-induced mammary carcinomas via microarray analysis.
Prolactin is a well recognized mitogen in the breast [15]. In the rat, the short-form of the PRLR (PRLRshort) has been considered an inhibitor and PRLRlong an activator of the Jak2/STAT5 pathway for differentiation and lactation [30–33]. However, there are several reports suggesting that PRLRshort may be mitogenic in certain cell types. For example, Das and Vonderhaar [34] demonstrated that PRLRshort can induce a mitogenic signal in NIH-3T3 cells possibly via the MAP kinase pathway. In addition, in heterozygous PRLR+/- mice, PRLRshort was able to induce proliferation in the mammary gland [35]. Furthermore, Russell and Richards [36] have suggested the possibility that PRLRshort is important for STAT5a phosphorylation and turnover. Our findings support the possibility that elevated levels of both PRLRlong and PRLRshort facilitate proliferation in carcinomas with high pY-STAT5a.

There are a growing number of studies concerning the expression of Stat5 in human breast cancer [4,11,37,38]. The results of the present study support a role for activated Stat5a in the proliferation and survival of rat mammary gland carcinomas. In agreement with the findings of the present study, Ren et al. [39] demonstrated that loss of STAT5a in breast cancer cells results in inhibition of tumorigenesis and an increase in the apoptotic index. While the role specifically of STAT5a in human breast cancer is still unclear, recent evidence suggests that STAT5 activation distinguishes breast cancer patients with favorable prognosis [4,40]. PhIP-induced rat mammary gland carcinomas are not generally metastatic and have
features similar to human breast cancers with good prognosis. Further studies of Stat5a activation in the PhIP-induced rat mammary gland cancer model is expected to provide insight into the role of Stat5a in some types of human breast cancer.
References


