RESEARCH ARTICLE

Editorial Process: Submission:10/01/2018 Acceptance:04/14/2019

The Effect of P-Glycoprotein (P-gp), Nuclear Factor-Kappa B (*Nf-κb*), and Aldehyde Dehydrogenase-1 (*ALDH-1*) Expression on Metastases, Recurrence and Survival in Advanced Breast Cancer Patients

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Abstract

Objective: To investigate the level of three drug resistance proteins; P-glycoprotein 1 (P-gp), nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*) and aldehyde dehydrogenase isoform 1 (*ALDH1*) expression and their relationship to metastasis, recurrence and survival in advanced breast cancer patients that received neoadjuvant chemotherapy. **Methods:** This study is a combination of prospective and retrospective cohort study involving one hundred and thirty one cases of advanced stage invasive breast cancer that have received neoadjuvant chemotherapy. Initial biopsy specimens (incisional biopsy or core biopsy) were taken from paraffin blocks. Immunohistochemistry (IHC) was used to detect P-gp, *NF-κB*, and *ALDH1* expression. Prospectively analysed patients were followed for five years and evaluated for recurrence and death. **Results:** The expression of P-gp has no significant statistical correlation to metastases (p = 0.659), recurrence (p = 0.862) and survival (p = 0.835) in advanced stage breast cancer patients who received neoadjuvant chemotherapy. Similarly, ALDH1 was not correlated to metastases (p=0.120), recurrence (p = 0.186) and survival (p = 0.254) statistically. We found that *NF-κB* expression showed a significant correlation to metastases (p=0.004), recurrence (p = 0.016) and overall survival (p = 0.041) in advanced stage breast cancer patients after neoadjuvant chemotherapy. **Conclusion:** *NF-κB* expression is a potential marker that can be used to assess or to predict increasing risk of metastases, recurrence and survival in advanced stage breast cancer patients who receive neoadjuvant chemotherapy.

Keywords: Breast cancer- neoadjuvant chemotherapy- *P-gp- NF-κB- ALDH1*

Asian Pac J Cancer Prev, **20 (5)**, 1511-1518

Introduction

GLOBOCAN data issued by the International Agency for Research on Cancer World Health Organization showed that the incidence of new breast cancer in Indonesia were 48.998 cases (16.4 %) (GLOBOCAN, 2012). Breast cancer is one of the most prevalent malignancies in women; the 5-year prevalence rate was 187.7 per 100,000 populations with a mortality rate of 16.6 for every 100,000 cases. Based on the database of health research in 2013 issued by Indonesia Ministry of Health, cervical cancer and breast cancer is a disease with the highest prevalence, which amounted to 0.8 ‰ for cervical cancer and 0.5 ‰ for breast cancer (Kesehatan, 2013). Estimation for absolute number of breast cancer cases was 61.682. The domestic health survey by the Ministry of Health showed that the death rate for breast cancer is increasing, from 1.4% in 1972, 3.4% in 1980, 4.3% in 1986 and to 4.4% in 1992. The prevalence of locally advanced breast cancer in Indonesia is estimated to be higher than neighbouring countries. Currently, there are limited data describe the prevalence and outcome of locally advanced breast cancer. Ramli (2015) found that from all cases of local advanced stage breast cancer, 23% were operable stage IIIA and 40% were inoperable stage IIIB.

Approximately 70% of breast cancer patients failed to achieve complete pathological response after neoadjuvant chemotherapy, whereas that response is representative of long-term survival (Chollet et al., 1997; Smith et al., 2002). Patients with advanced stage breast cancer that poorly respond to chemotherapy are at higher risks of local and systemic recurrence, as well as poor long term disease-free survival rate. Non-optimal or poor response to neoadjuvant chemotherapy was postulated due to the combination of chemoresistant mechanisms. Such mechanisms include over expression of ATP binding cassette (ABC) transporter, apoptosis dysregulation, and possibly excess number of cancer stem cells (Weldon et al., 2001; Kuo,

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2007; Kida et al., 2016). The chemoresistant process might involve more than one of these mechanisms. This study investigated all three components: P-glycoprotein (P-gp), which is part of ABC transporter, expression of nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*) that plays a role in the regulation of apoptosis and aldehyde dehydrogenase isoform 1 (*ALDH1*) which a functional marker of cancer stem/progenitor cells as critical components related to breast cancer metastases, recurrence and patient survival.

Materials and Methods

Patient Selection

This is a multicentre study that combines both prospective and retrospective cohort data of one hundred and thirty one cases of advanced stage invasive breast cancer within the period of January 2008 to December 2011. The data were acquired from 5 hospitals in Dr. Margono Soekarjo hospital in Purwokerto, and Dr. Hasan Sadikin hospital in Bandung. Dr. Kariadi Hospital, Telogorejo hospital, Sultan Agung hospital, Dr. Roemani hospital in Semarang.

The inclusion criteria were patients diagnosed with advanced, invasive breast cancer stage IIB, stage IIIA, IIIB and IIIC according to AJCC 7th edition TNM staging system. Those with inflammatory breast cancer (T4d), history of previous treatment (medical/non-medical), incomplete medical records, incomplete follow-up, missing paraffin block or histopathological results, incomplete treatment or dropped-out were excluded.

All patients included in the study underwent initial biopsy (open incisional or core biopsy) to obtain histopathological grading. Afterwards neoadjuvant chemotherapy with FAC regimen: doxorubicin 50 mg/m2; fluorouracil 500 mg/m2; and cyclophosphamide 500 mg/m2 given on day 1, every 3 weeks was given. After three to four cycles of chemotherapy, all patients underwent either mastectomy or breast conserving surgery. Some evaluation of pathological responses was taken from paraffin-embedded mastectomy or lumpectomy tissue. All patients were followed up for 5 years, all evidence of recurrences both local and systemic were recorded, noting the location of the recurrences. In that time span, any evidence of death by any reasons was also recorded.

Immunohistochemistry (IHC)

P-gp expression was preformed using Avidin-Biotin Peroxidase technique on a tissue section from paraffin blocks. The sections were placed in a dry oven 37ºC for one night, deparaffinised in xylene and rehydrated in serial ethanol. Endogenous peroxidase activity was blocked with 50 mL of methanol with 3% hydrogen peroxide. Slides were then incubated in 3% horse serum (Vector Laboratories, Burlingame, CA) at a temperature of 37ºC for 20 minutes, then incubated in primary antibody at a temperature of 37ºC for 30 minutes and washed in phosphate buffered saline (PBS). Rabbit polyclonal antibodies to PGP ab103477 (Abcam, Cambridge, UK) were used. Biotinylated secondary antiserum dropped on the slide, and then incubated at 37ºC for 30 minutes and washed in PBS. Slides were then incubated with Vector Elite Avidin-Biotin Complex (Vector) at room temperature for 30 minutes and washed in PBS. Diaminobenzidine (DAB) was used as peroxidase substrate to form a color. Slides were then incubated in DAB 1 g/mL, 45 mL of PBS and 3% hydrogen peroxide for 2-7 minutes, counterstained with aqueous hematoxyline for 2 minutes, then washed in PBS and water flow. Multidrug-resistant cell-C4 and KB-8-5, and multidrug-sensitive cells KB-3-1 was used as positive and negative controls (Fojo et al., 1987; Romano et al., 2009).

Expression of *NF-κB* was assessed from 4 μm slide from paraffin blocks of tumor tissue, deparaffinised in xylene and several concentration of alcohol. Endogenous peroxidase was blocked with hydrogen peroxide 0.03% for 5 minutes. Slides were then washed with Tris-buffered saline containing 0.1% Tween 20 at pH 7.6 and incubated with primary antibody using Rabbit polyclonal to NFkB p65-Chip Grade ab7970 (Abcam, Cambridge, UK) at a dilution of 1:600. The sections were then treated with 3,3'-diaminobenzidine as chromogen for 5 minutes and counterstained with haematoxylin. Slides were then washed with water, dehydrated and covered with a glass coverslip.

Expression of ALDH1 was evaluated by the method of avidin-biotin-peroxidase using anti-ALDH1. To distinguish tumor cells with macrophage-positive ALDH1 and ALDH1-positive, double immunohistochemistry staining using ALDH1 and CD68 (a marker for macrophages) were used. 3 μm-thick paraffin section was incubated with antibody Concentrated Monoclonal Antibody Aldh1a1 (Biocare Medical, Concord, USA). ALDH1 positive expression was confirmed by sections of the same tissue that performed staining antibodies Monoclonal Mouse Anti-Human CD68 Clone KP1 Ready-to-Use (Dako, Glostrup, Denmark)

Statistical analysis

The Microsoft Excel for Mac 2011 was used to create a database for collected information. And statistic analysis was carried through IBM SPSS Statistics 20. The survival data was statically analyzed using Kaplan Meier method to determine the effect of *P-gp*, *NF-κB*, and *ALDH1* against recurrences, metastases, and death (overall survival). Proportional Hazards (Cox) Regression was used to examine the correlation of each factor independently with survival data, by controlling confounding variables. P value <0.05 was considered as significant. A p value of <0.05 was considered to be statistically significant

This research was carried out with reference to the principles mentioned in the Helsinki Declaration (2000) and the project was approved by The Medical And Health Research Ethics Committee (MHREC) Ministry of National Education Faculty of Medicine Gadjah Mada University Ref : KE/FK/195/EC.

Results

The average age of patients included in this study was 48.48 years old (SD 9.57), with the youngest was 27 years old and the oldest was 76 years old. The expression

of P-glycoprotein mostly was negative in this study population (66.4%). Only 33.6% of them were positive expression (Table 1)

In the positive population, 58.9% of them scores +1; only 22.6% are +3 (intense staining). Similar results were published by Campos et al., (2005). Their study on stage III breast cancer that received neoadjuvant chemotherapy showed P-glycoprotein positivity as much as 23.86%, and it was related with the worse prognostic of the disease. But higher rate of positivity was mentioned by Chintamani et al., (2005), which got as high as 52%. Of the entire study population, the majority (70.2%) expressed the *NF-κB* protein. Among the positive population, 63% of them include in the group with a population of cancer cells express the *NF-κB* more than 50%. NFκB positivity rate in this study are far greater than other studies. Such as the study by Montagut et al., (2006), which got a positive number of only 13%. This obvious difference is due to the difference of classification in the positive NFκB assessment. Only 20.3% of the study population expressed *ALDH-1*. Among the positive expression, as much as 13.7% of expressed < 10% with weak-to-strong intensity. In the series of studies on 108 primary breast cancer patients, Tanei et al., (2009) found a proportion of 19% *ALDH-1* positive expression, while 81% were negative. This result is similar to that obtained by this study.

There were 58 report of tumor recurrence (Figure 1).

Table 1. The Frequency of P-gp, NFκB, and ALDH1 Expression

	n = 131	%
P-gp1		
Negative	87	66.4
+1 (<25% positive cells)	26	19.8
+2 (25-50% positive cells)	8	6.1
+3 (>50% positive cells)	10	7.6
NF-kB2		
Negative	39	29.8
< 50% weak	19	14.5
< 50% moderate	8	6.1
< 50% strong	7	5.3
> 50% weak	15	11.5
> 50% moderate	15	11.5
> 50% strong	28	21.4
ALDH13		
Negative	107	81.7
< 10% weak	8	6.1
< 10% moderate	8	6.1
< 10% strong	2	1.5
10-50% weak	1	0.8
10-50% moderate	4	3.1
10-50% strong	1	0.8

[1] P-gp, P-glycoprotein. Results of +1, +2 and +3 classified as positive expression; 2 NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells. Result other than "negative" considered as positive expression; [3] ALDH1, aldehyde dehydrogenase isoform 1. Results other than "negative" considered as positive expression.

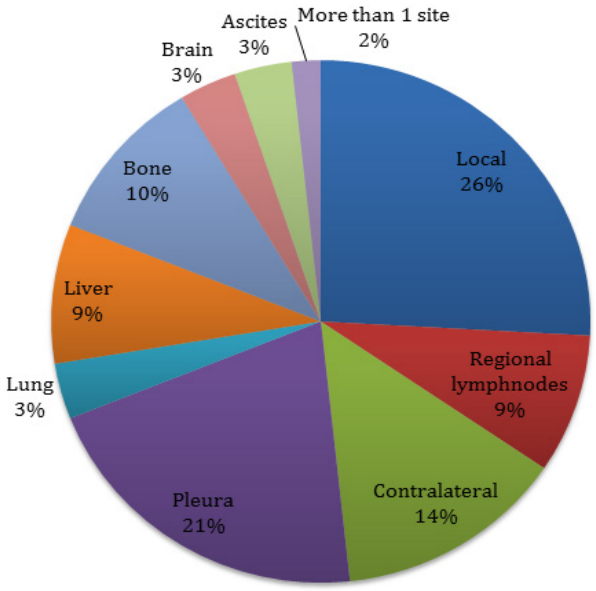


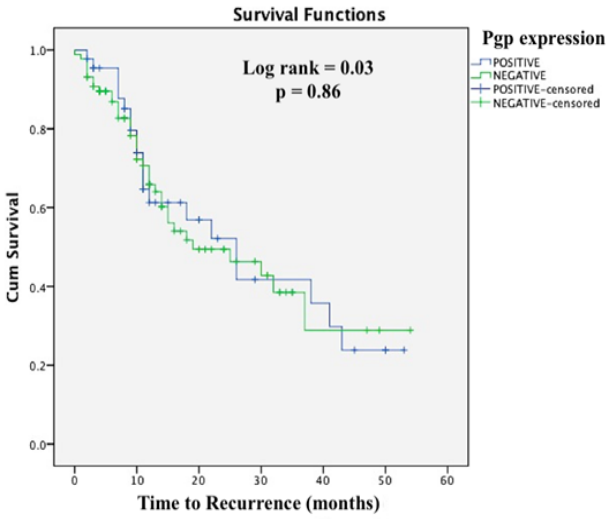
Figure 1. Location and Frequency Tumor Recurrence

The most frequent recurrence sites were local recurrence (25.9%), followed by pleura (20.7%), and bone (10.3%). Only 1 patient experienced tumor recurrence in more than 1 location. Autopsies performed in the United States between 1943 and 1977 concluded that the metastatic pattern did not change within the 35-year range (Lee, 1983). The incidence of the six most common sites is the lungs, bones, lymph nodes, liver, pleura, and adrenal glands. Similar observations are also found in Europe. An observational study performed by Iqbal et al., (2010) found that the most frequent recurrence was loco regional, followed by visceral, and bone recurrence.

There was no correlation between *P-gp* expression with age, menopausal status, histopathological grade, lymphovascular invasion, or molecular subtype. Meanwhile *NF-κB* expression had strong correlation with lymphovascular invasion (p = 0.015), in which positive *NF-κB* expression is associated with positive LVI, whereas negative *NF-κB* expression is associated with negative LVI. There was small association with histopathological grade, although not significant (p= 0.094), positive *NF-κB* expression was associated with high-grade histopathology, whereas negative *NF-κB* expression was associated with low and intermediate grade (Table 2)

ALDH-1 expression correlates with age (p = 0.023), positive expression is related to young age (≤ 45 year old), whereas negative expression is associated with older age (> 45 year old). *ALDH-1* expression is also significantly correlated with molecular subtype (p = 0.000). The positive expression is higher in HER2 subtype, whereas the negative expression is higher in Luminal and Triple Negative subtypes. In a population of patients with negative ER, 66.3% of them showed positive NFκB expression (p = 0.311). In the population of patients with positive HER2, 76.9% showed positive NFκB expression (p = 0.477). These results are in accordance with that stated by Biswas et al., (2003) and Karin et al., (2002) that the activation of NFκB in human breast cancer is found mostly in the ER-negative subtype, specifically those that

(a)



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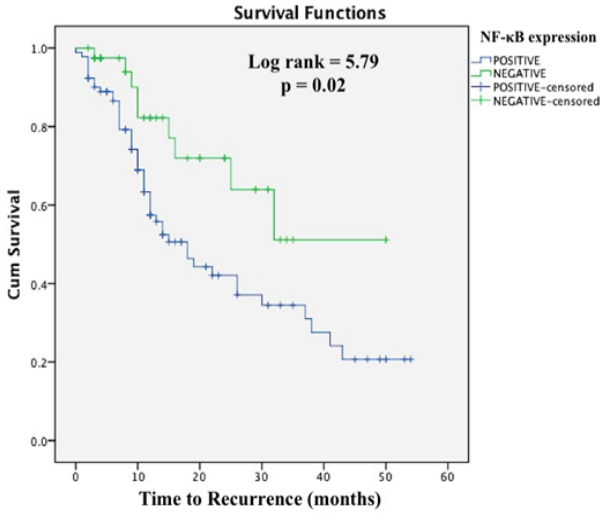

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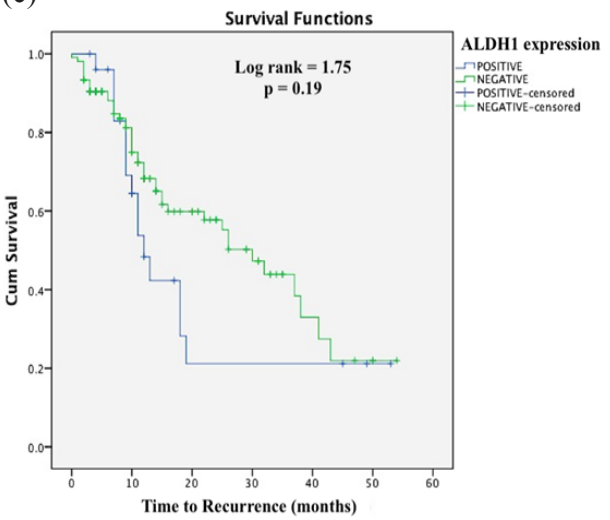
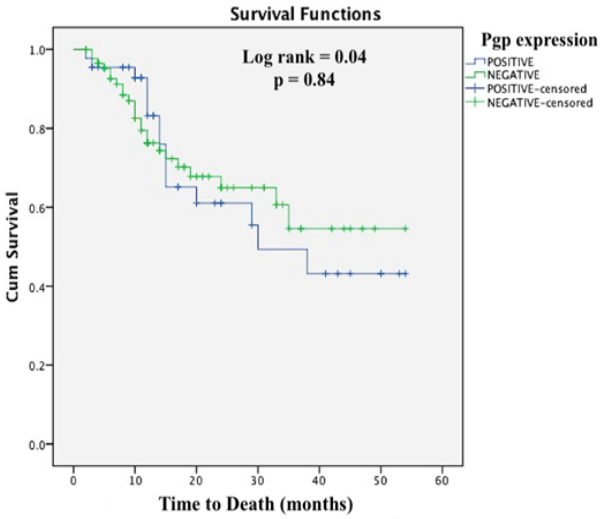


Figure 2 a-c. Diagram of Survival Function for Investigated Prognostic Factors Related to Time of Recurrence

(a)



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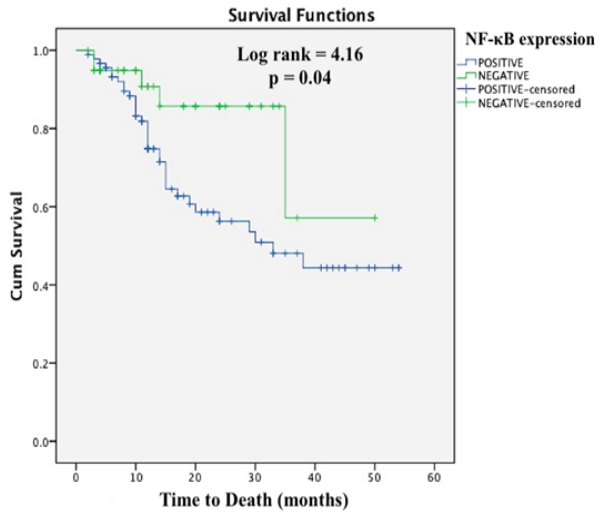

(c)

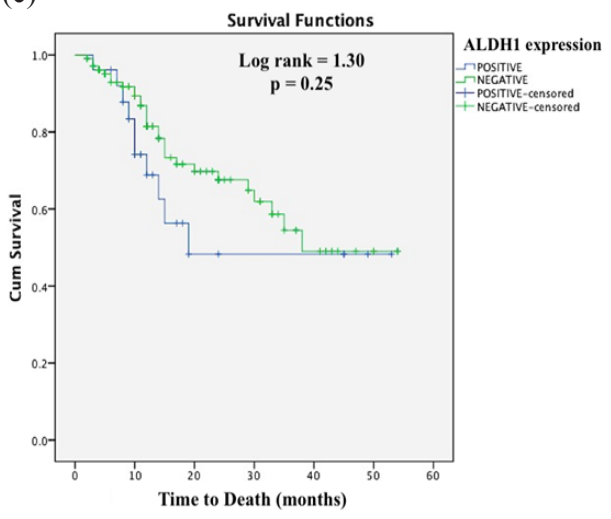

Figure 3 a-c. Diagram of Survival Function for Investigated Prognostic Factors Related to Time of Death.

demonstrate members of the EGF family of receptors including HER-2.

This study also found that HER2 positive expression had a tendency to express positive NFκB (76% vs 23.1%), although it was not statistically significant. These results are similar to study conducted by Montagut et al., (2006).

It is thought that HER2 might be involved in NFκB activation via the phosphotidyl inositol 3-kinase/Akt intracellular pathways (Biswas et al., 2000; Zhou et al., 2000; Pianetti et al., 2001).

Positive NFκB expression was significantly correlated with metastases, recurrence, and survival (p=0.004; p=0.16; and p=0.041) (Table 3). One of the reasons why NFκB significantly influences the metastatic processes is that metalloproteinases, urokinase-type plasminogen activator, and cytokines are upregulated by NFκB in highly metastatic, aggressive breast cancer lines (Helbig et al., 2003). It is also seen to increase motility of breast cancer cells by directly up-regulating the expression of CXCR4. Whereas ALDH1 as a prognostic marker, although statistically not significant showed the relationship with the long-term results. Where the negative expression of

Table 2. Frequency of Other Identified Prognostic Factors Related to the Expression of P-gp, NFκB, and ALDH-1

	P-gp		NFκB		ALDH1	
Prognostic factors	Positive (n=44)	Negative (n=87)	Positive (n=91)	Negative (n=40)	Positive (n=26)	Negative (n=105)
Age (y.o.)						
≤ 45 (young)	13 (26.5)	36 (73.5)	36 (73.5)	13 (26.5)	15 (30.6)	34 (69.4)
> 45 (old)	31 (37.8)	51 (62.2)	55 (67.1)	27 (32.9)	11 (13.4)	71 (86.6)
	p=0.251		p=0.557		p=0.023	
Menopausal status						
Pre (<50 y.o.)	21 (29.2)	51 (70.8)	50 (69.4)	22 (30.6)	16 (22.2)	56 (77.8)
Post (≥50 y.o.)	23 (39.0)	36 (61.0)	41 (69.5)	18 (30.5)	10 (16.6)	49 (83.1)
	p=0.268		p=1.00		p=0.514	
Grade						
3	28 (30.1)	65 (69.9)	69 (74.2)	24 (25.8)	20 (21.5)	73 (78.5)
1 and 2	16 (42.1)	22 (57.9)	22 (57.9)	16 (42.1)	6 (15.8)	32 (84.2)
	p=0.223		p=0.094		p=0.630	
Lymphovascular invasion						
Yes	36 (36.7)	62 (63.3)	74 (75.5)	24 (24.5)	19 (19.4)	79 (80.6)
No	8 (24.2)	25 (75.8)	17 (51.5)	16 (48.5)	7 (21.2)	26 (78.8)
	p=0.209		p=0.015		p=0.805	
Molecular subtype						
Luminal A	14 (45.2)	17 (54.8)	20 (64,5)	11 (35.5)	7 (22.6)	24 (77.4)
Luminal B	9 (28.1)	23 (71.9)	26 (81.3)	6 (18.8)	3 (9.4)	29 (90.6)
Triple Negative	16 (32.0)	34 (68.0)	31 (62.0)	19 (38.0)	6 (12.0)	44 (88.0)
HER2	5 (27.8)	13 (72.2)	14 (77.8)	4 (22.2)	10 (55.6)	8 (44.4)
	p=0.454		p=0.226		p=0.000	
ER expression						
Positive	17 (40.5)	25 (59.5)	32 (76.2)	10 (23.8)	9 (21.4)	33 (78.6)
Negative	27 (30.3)	62 (69.7)	59 (66.3)	30 (33.7)	17 (19.1)	72 (80.9)
	p=0.322		p=0.311		p=0.816	
PR expression						
Positive	16 (33.3)	32 (66.7)	35 (72.9)	13 (27.1)	6 (12.5)	42 (87.5)
Negative	28 (33.7)	55 (66.3)	56 (67.5)	27 (32.5)	20 (24.1)	63 (75.9)
	p=1.000		p=0.560		p=0.119	
HER2 expression						
Positive	7 (26.9)	19 (73.1)	20 (76.9)	6 (23.1)	12 (46.2)	14 (53.8)
Negative	37 (35.2)	68 (64.8)	71 (67.6)	34 (32.4)	14 (13.3)	91 (86.7)
	p=0.493		p=0.477		p=0.001	
Ki67 expression						
> 13%	18 (33.3)	36 (66.7)	41 (75.9)	13 (24.1)	11 (20.4)	43 (79.6)
Neg and < 13%	26 (33.8)	51 (66.2)	50 (64.9)	27 (35.1)	15(19.5)	62 (80.5)
	p=1.000		p=0.247		p=1.000	

P-gp, P-glycoprotein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ALDH1, aldehyde dehydrogenase isoform 1; y.o, years old; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; Ki67, marker of proliferation Ki-67

Table 3. Correlation between Prognostic Factors Reviewed Including PGP, NFKB, ALDH1 with Metastases, Recurrence and Survival

		Metastases			Recurrence			Survival		
		Yes	No	p	Yes	No	p	Dead	Alive	p
P-gp	+ve	12	32	0.659	21	23	0.862	15	29	0.835
	-ve	27	60		37	50		24	63	
NFκB	+ve	34	57	0.004	49	42	0.016	34	57	0.041
	-ve	5	35		9	31		5	35	
ALDH1	+ve	11	15	0.12	15	11	0.186	10	16	0.254
	-ve	28	77		43	62		29	76	

P-gp, P-glycoprotein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ALDH1, aldehyde dehydrogenase isoform; +ve, positive expression; -ve, negative expression

ALDH1 was found more in the group that metastases did not occur, recurrence did not occur, and also have longer survival (p=0.120; p=0.186; p=254).

Expression of *P-gp* has no effect on recurrence time (p = 0.86). On the other hand, expression of *NF-κB* has a relationship to the recurrence time (p = 0.02). Where a tumor with a positive expression has a higher recurrence rate. This study also obtained the result that the presence of intratumoral *ALDH1* cancer stem cells was associated with recurrence time although statistically it was not significant (p = 0.19). It can be seen that in the first twenty months, patients with positive expression of *ALDH1* showed a higher recurrence rates. A somewhat different result were delivered by Resetkova et al., (2010) they found that positive expression of *ALDH1* did not correlate with disease free survival.

The *P-gp* expression was not related to overall survival (p = 0.84). However the expression of NFκB was significantly associated with overall survival (p = 0.04), where positive expression had a worse overall survival. The expression of *ALDH1* appears to have a relationship to overall survival, although it was not statistically significant (p = 0.25). Where at least in the first forty months, the positive expression of *ALDH1* showed a worse overall survival. Resetkoval et al., (2010) found different results reporting *ALDH1* expression did not correlate with overall survival. However, Ginestier et al., (2007) and Neumeister et al., (2009) reported similar results showing stronger expression of *ALDH1* is related with poorer long-term survival.

Discussion

This study has found no significant correlation between P-pg with recurrences, metastases, and long-term survival of advanced breast cancer patients. This finding is similar to the results reported by Pinedo and Giaccone (1995) which states that in breast cancer, even the positive expression of P-pg is more commonly found in locally advanced tumors, however the correlation has not been observed. They stated that perhaps *P-gp* is more a marker of tumor aggressiveness than of response to the treatment.

As postulated at the beginning that the expression of ALDH1 is related to a worse prognosis, this study seems to show the relationship between *ALDH1* expression with recurrences, metastases and long-term survival, although these results were not statistically significant. Ginestier et al., (2007) in their research had shown that *ALDH1* is a better marker in identifying breast cancer stem cells. Abraham et al., (2005) and Ginestier et al., (2007) have proven the existence of breast cancer stem cells and their relation to a biologically aggressive phenotype. ALDH1 positivity generally also expresses high level of ABC transporter, so it is estimated to be resistant to chemotherapy. Different results in this study can be cause by various conditions. For example by different staining methods, or the weakness arising form paraffin block production from the beginning of the preparation to the staining phase.

Several studies have been conducted to identify reliable predictive and prognostic factors to determine therapeutic response and long-term results on locally advanced breast cancer. Classical clinical-pathological parameters that have been studied, including age, tumour size, nodal status, nuclear tumour grade, hormonal receptor status, HER2 expression, Ki67 expression, etc. It is estimated that a combination of these factors in breast cancer management has a higher predictive and prognostic value. But the role of these factors in term of prognostic value, especially in locally advanced breast cancer is often vague. The prognostic value of NFκB in several types of cancer has been previously reported (Lessard et al., 2003; Fradet et al., 2004; Ross et al., 2004; Domingo-Domenech et al., 2005; Xia et al., 2014).

Our study found a significant correlation between NFκB positivity and poorer prognosis in terms of recurrences, metastases, and long-term survival. These results are in line with what was conveyed by Dolcet et al., (2005) that tumors with constitutive NFκB activation usually show increased resistance to chemotherapy, and ultimately affect long-term result. It was also reported that NFκB might induce expressions of the multidrug resistance P-glycoprotein. Some preclinical studies also have shown activation of the NFκB pathway by different chemotherapy agents, including anthracyclines and taxanes (Das and White, 1997; Bottero et al., 2001; Ho et al., 2005). This unfavourable effect also results from the activation of anti-apoptotic genes by NFκB (Wang et al., 1996; Karin, 2009; Wang et al., 2012).

The role of NFκB expression as prognostic factor in breast cancer especially the locally advanced stage seems very promising. Hence it can be applied in patient

management, along with other established prognostic factors. Perhaps from a therapeutic side it does not have clear clinical benefits, but its role as a new prognostic factor will help us classify patients into groups with good or worse long-term result predictions. Therefore we can plan the steps needed both sociopsychologically and medically including therapeutic planning that might use. Hopefully this research can increase our knowledge, especially in breast cancer management.

Statement conflict of Interests

None declared.

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