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# ALDH1 expression is enriched in breast cancers arising in young women but does not predict outcome

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**Background:** Tumours arising in younger women appear to be biologically more aggressive and tend to have a poorer outcome. Being relatively resistant to conventional treatments, breast cancer stem cells (CSCs) have been postulated as a possible cause of disease recurrence after treatment. In this study, we used ALDH1 as a CSC marker and determined whether ALDH1 expression correlated with clinical outcome in young women with breast cancer.

**Methods:** The expression of ALDH1 was evaluated through immunohistochemistry on microarrayed cores obtained from 141 consecutive patients up to 35 years of age.

**Results:** The expression of ALDH1 was observed in 25% (35 of 141) of tumours, in a median of 5% of cells. Younger women were 14 times more likely to have ALDH1-positive tumours ( $P < 0.01$ , OR 14.4, 95% CI 4.34–48.09). The ALDH1 correlated independently with ER negativity ( $P = 0.01$ , OR 0.33, 95% CI 0.15–0.77). There was no correlation with disease recurrence or breast cancer-related deaths.

**Conclusion:** In younger women, ALDH1 was more highly expressed, and it correlated with ER negativity. It, however, did not predict survival in this study.

Breast cancers arising in young women have a comparatively poorer outcome (El Saghir *et al*, 2006). These tumours are often of high tumour grade, with lymphovascular invasion and nodal involvement, and a significant number are triple negative, lacking in oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) expression (Fernandopulle *et al*, 2006; Bauer *et al*, 2007). Triple-negative tumours have a propensity for early and rapidly progressive metastatic recurrence and, as a group, respond less favourably to chemotherapy (Dent *et al*, 2007; Liedtke *et al*, 2008; Tan *et al*, 2008).

The relative abundance of breast cancer stem cells (CSCs) in triple-negative tumours may be a possible explanation (Honeth *et al*, 2008; Idowu *et al*, 2012). The CSCs are largely quiescent

and thus less susceptible to conventional treatments. Aberrant expression of ATP-cassette binding transporters, anti-apoptotic factors and DNA repair enzymes further contribute to chemoresistance (Phillips *et al*, 2006; Shi and Harris, 2006). Aldehyde dehydrogenase 1 (ALDH1), identified as a breast CSC marker, has been associated with chemoresistance and poor prognosis (Pearce *et al*, 2005; Ginestier *et al*, 2007; Tanei *et al*, 2009). As ALDH1 was reportedly upregulated in triple-negative tumours and tumours arising in African women, which share several similarities with those arising from young women, we hypothesise that a relative abundance of ALDH1-positive CSCs may account for the poor outcome in younger women despite aggressive treatment (Ginestier *et al*, 2007; Nalwoga *et al*, 2010; Zhou *et al*, 2010; Ohi *et al*, 2011; Idowu *et al*, 2012). In this study, we used ALDH1

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to identify the breast CSC subpopulation and evaluated whether ALDH1 expression correlated with clinical outcome.

## MATERIALS AND METHODS

**Patient and tumour characteristics.** Microarrayed tissue comprising 1 mm cores were collected from 141 consecutive women  $\leq 35$  years old who underwent surgery at the Singapore General Hospital from 1993 to 2009. Two separate cores were obtained from each tumour specimen; necrotic regions were avoided. This study has Ethical Committee approval (2011/433/B). Median patient age was 32 years (range 19 to 35 years). Although the ethnic make-up was reflective of the local population, with Chinese women accounted for the majority (67%), these younger women were 4 times more likely to be Malay as compared with those diagnosed after 35 years of age ( $P < 0.01$ , OR 3.90, 95% CI 2.08–7.31). The majority of tumours (128 of 141, 91%) were classified as invasive ductal carcinoma of no special type (Table 1). Median pathological tumour size was 22.5 mm (range 5 to 155 mm), and median tumour grade, according to the modified Bloom and Richardson criteria, was 3. In all, 43% of tumours had nodal involvement; 55% were ER positive, 53% PR positive and 21% HER2 positive. All women underwent curative surgery and received adjuvant therapy according to the NCCN guidelines. None received neoadjuvant treatment. Over the follow-up period (median 70 months, range 18 to 221 months), 49 patients developed recurrent disease (13 with local recurrence alone) and 27 died of breast cancer-related causes.

Comparison was made with 145 women  $> 35$  years old diagnosed during the same period. Median age of this group was 67 years (range 36 to 89 years). Details are shown in Table 1. Similarly, the majority of women were of Chinese ethnicity (129 of 145, 89.0%). Most tumours were classified histologically as being of invasive ductal carcinoma of no special type (130 of 145, 89.6%), Median tumour size was 28 mm (range 2 to 190 mm), and median tumour grade was 2. Compared with tumours arising in younger women, these tumours were more likely to be of lower tumour grade and ER positive ( $P < 0.01$  and  $P < 0.01$ , OR 3.33, 95% CI 1.97–5.62 respectively). However, these tumours were found more likely to have lymphovascular invasion ( $P < 0.01$ , OR 7.67, 95% CI 4.50–13.07). Recurrence occurred in 33 patients (local recurrence alone in 4) and 32 patients died from breast cancer-related causes.

**Immunohistochemistry.** The expressions of ALDH1, ER, PR and HER2 were evaluated by immunohistochemistry. Primary antibodies used included rabbit monoclonal ALDH1 antibody (clone EP1933Y, 1:100 dilution, Abcam, Cambridge, UK), ER antibody (clone SP1, 1:50 dilution, RM-9101-R7), PR antibody (clone PgR636, 1:200 dilution, Dako M3569, Glostrup, Denmark) and HER2 antibody (clone SP3, 1:200 dilution, RM-9103-R7). Briefly, 4  $\mu$ m-thick sections were dewaxed in xylene and rehydrated in graduated ethanol solutions. Sections were subjected to heat-induced antigen retrieval (0.01 M Tris-0.001 M EDTA, pH 9, at 98 °C in a microwave) and then run on the Dako Autostainer Plus. The Dako Envision Detection kit (K5007) was used and slides were counterstained in Mayer's haematoxylin (Dako S3309). Normal liver was used as positive control. The ALDH1 staining was scored with respect to staining intensity (0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining) and the percentage of tumour cells stained. Various thresholds for positive staining have been reported (Ginestier *et al*, 2007; Chang *et al*, 2009; Nalwoga *et al*, 2010; Sullivan *et al*, 2010). Tumours with positive staining in the cytoplasm in  $> 10\%$  of cells were considered positive in this analysis. For ER and PR, nuclear staining in at least 1% of tumour cells was considered positive; for HER2, strong membranous staining in at least 30% of tumour cells was considered positive.

**Table 1.** Comparison of standard clinicopathological parameters in women  $> 35$  years of age and those  $\leq 35$  years of age ( $n = 286$ )

|                                      | Women $> 35$<br>years of age<br>( $n = 145$ ) | Women $\leq 35$<br>years of age<br>( $n = 141$ ) | P-value  |
|--------------------------------------|---|--|----------|
| Ethnicity                            |   |  | $< 0.01$ |
| Chinese                              | 129   | 95   |          |
| Malay                                | 8   | 20   |          |
| Indian                               | 2   | 7  |          |
| Others                               | 6   | 19   |          |
| Tumour histology                     |   |  | 0.75     |
| IDC                                  | 130   | 128  |          |
| Non-IDC                              | 15  | 13   |          |
| Median pathological tumour size (mm) | 22.5 (5.0–115.0)                              | 28.0 (2.0–190.0)                                 | 0.05     |
| Tumour grade                         |   |  | $< 0.01$ |
| 1                                    | 23  | 11   |          |
| 2                                    | 51  | 43   |          |
| 3                                    | 63  | 86   |          |
| Lymphovascular invasion              |   |  | $< 0.01$ |
| Present                              | 115   | 47   |          |
| Absent                               | 30  | 94   |          |
| Nodal status                         |   |  | 0.25     |
| Positive                             | 64  | 44   |          |
| Negative                             | 63  | 59   |          |
| ER status                            |   |  | $< 0.01$ |
| Positive                             | 116   | 77   |          |
| Negative                             | 29  | 64   |          |
| PR status                            |   |  | 0.81     |
| Positive                             | 72  | 72   |          |
| Negative                             | 73  | 69   |          |
| HER2 status                          |   |  | 0.90     |
| Positive                             | 32  | 32   |          |
| Negative                             | 113   | 109  |          |

Abbreviations: ER = oestrogen receptor; HER2 = human epidermal growth factor receptor-2; IDC = invasive ductal carcinoma; Non-IDC = tumour histologies other than invasive ductal carcinoma; PR = progesterone receptor.

**Statistical methods.** Correlation analyses were made using the  $\chi^2$  test or Fisher's exact test with GraphPad Prism version 4 (GraphPad software Inc., San Diego, CA, USA). Cox proportional hazard regression model was performed with the Stata package release 8.1 (Stata Corporation, College Station, TX, USA). A two-tailed  $P$ -value test was used and a  $P$ -value of  $< 0.05$  was considered statistically significant.

## RESULTS

Of the 141 tumours, 35 (25%) stained positive for ALDH1 (Figure 1). Median staining intensity was weak (score 1), and ALDH1 staining was completely absent in 69 tumours (49%). In most tumours, ALDH1 staining was observed only in a median of 5% of tumour cells and only 7 tumours had  $> 50\%$  of cells staining positive for ALDH1. The ALDH1 was observed mainly in the cytoplasm. Scattered foci of staining were observed in the stroma,

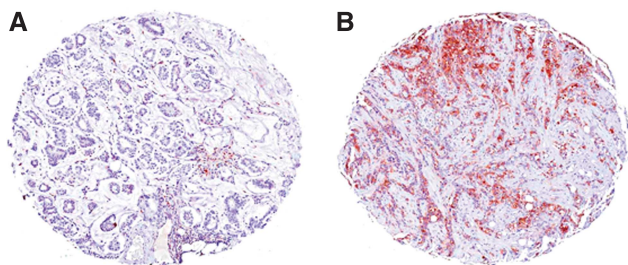


Figure 1. Immunohistochemistry of ALDH1 in invasive breast carcinoma. (A) negative staining; (B) positive cytoplasmic staining in > 10% of tumour cells.

although nuclear staining was largely absent. Among the 145 women >35 years old, only 3 tumours stained positive for ALDH1. Intensity of staining was weak (score 1) in all cases and 10% to 20% of cells stained positive for ALDH1. Young women were 14 times more likely to have ALDH1-positive tumours ( $P < 0.01$ , OR 14.4, 95% CI 4.34–48.09).

Among the young women, tumours expressing ALDH1 were 3 times more likely to be ER negative ( $P < 0.01$ , OR 3.03, 95% CI 0.15–0.73) and PR negative ( $P < 0.01$ , OR 3.52, 95% CI 0.12–0.65; Table 2). The ALDH1-positive tumours tended to be smaller, of high tumour grade and HER2 positive, but these did not reach statistical significance ( $P = 0.11$ ,  $P = 0.24$  and  $P = 0.62$ , respectively). There was no correlation with age, ethnicity, tumour histology, lymphovascular invasion and nodal status ( $P > 0.05$ ). On multivariate analysis, ER negativity was independently correlated with ALDH1 expression ( $P = 0.01$ , OR 0.33, 95% CI 0.15–0.77; Table 3). The expression of ALDH1 did not correlate with disease recurrence, whether local or distant or breast cancer-related deaths, and neither was there an association with the 5-year disease-free survival ( $P > 0.05$ ; Table 2 and Figure 2).

## DISCUSSION

In this study, we have found ALDH1 expression to be more common in tumours arising in younger women up to 35 years of age. A recent study also reported similar findings (Mieog *et al*, 2012). Other studies have not found such a correlation, but variations in age stratifications preclude direct comparisons (Gong *et al*, 2010; Nalwoga *et al*, 2010). Preferential upregulation of ALDH1 may account for the biological differences between tumours in younger and older postmenopausal women. Genes involved in stem cell biology were among the genes found to be differentially expressed among young women in a large multi-centric genomic analysis that included data from Singapore (Anders *et al*, 2008). We chose to focus on ALDH1 expression because ALDH1 identifies a highly tumorigenic subpopulation; tumours could be generated with as few as 20 ALDH1-positive CD44+/CD24– cells, whereas a similar effect was not observed even with more than 50 000 ALDH1-negative CD44+/CD24– cells (Ginestier *et al*, 2007).

In our study, we too observed that ALDH1-positive cells constituted only a small portion of the tumour population (Ginestier *et al*, 2007). The association with hormone unresponsiveness has been reported previously (Ginestier *et al*, 2007; Nalwoga *et al*, 2010; Zhou *et al*, 2010; Tsang *et al*, 2012). Although ER- and PR-negative tumours tend to be more aggressive, ALDH1 expression did not correlate with recurrence or death, and failed to predict survival, in our study. A recent report found CSCs, identified using ALDH1 and CD44/CD24 expression, to identify a poor prognostic subgroup among luminal-type cancers (Tsang *et al*, 2012). Several other studies have reported a poor

Table 2. Correlation analyses of ALDH1 expression and standard clinicopathological parameters and outcome ( $n = 141$ )

|  | ALDH1 positive (n = 35) | ALDH1 negative (n = 106) | P-value |
|--|-------------------------|--------------------------|---------|
| Median age (years)                           | 33 (23–35)              | 32 (19–35)               | 0.76    |
| Ethnicity                                    |                         |                          | 0.71    |
| Chinese                                      | 22                      | 73                       |         |
| Malay  | 5                       | 15                       |         |
| Indian                                       | 3                       | 4                        |         |
| Others                                       | 5                       | 14                       |         |
| Tumour histology                             |                         |                          | 0.06    |
| IDC  | 32                      | 96                       |         |
| ILC  | 0                       | 2                        |         |
| Mucinous carcinoma                           | 0                       | 7                        |         |
| Medullary carcinoma                          | 3                       | 1                        |         |
| Median pathological tumour size (mm)         | 20.0 (9.0–100.0)        | 26.0 (5.0–155.0)         | 0.11    |
| Tumour grade                                 |                         |                          | 0.24    |
| 1  | 1                       | 10                       |         |
| 2  | 10                      | 33                       |         |
| 3  | 23                      | 63                       |         |
| Lymphovascular invasion                      |                         |                          | 0.49    |
| Present                                      | 10                      | 37                       |         |
| Absent                                       | 25                      | 69                       |         |
| Nodal status                                 |                         |                          | 0.54    |
| Positive                                     | 11                      | 33                       |         |
| Negative                                     | 18                      | 41                       |         |
| ER status                                    |                         |                          | <0.01   |
| Positive                                     | 12                      | 65                       |         |
| Negative                                     | 23                      | 41                       |         |
| PR status                                    |                         |                          | <0.01   |
| Positive                                     | 10                      | 62                       |         |
| Negative                                     | 25                      | 44                       |         |
| HER2 status                                  |                         |                          | 0.62    |
| Positive                                     | 9                       | 23                       |         |
| Negative                                     | 26                      | 83                       |         |
| Recurrence                                   |                         |                          | 0.39    |
| Yes  | 11                      | 42                       |         |
| No   | 24                      | 64                       |         |
| Death  |                         |                          | 0.40    |
| Yes  | 5                       | 22                       |         |
| No   | 30                      | 84                       |         |
| Median 5-year disease-free survival (months) | 56.0 (13.0–60.0)        | 57.0 (10.0–60.0)         | 0.96    |

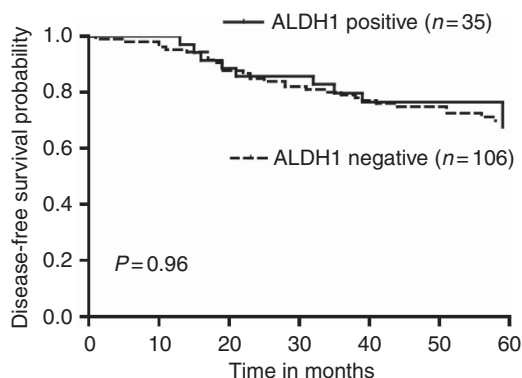
Abbreviations: ALDH1 = aldehyde dehydrogenase 1; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor-2; IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; PR = progesterone receptor.

outcome in ALDH1-rich tumours, but none of these studies stratified patients by age (Ginestier *et al*, 2007; Tanei *et al*, 2009; Resekova *et al*, 2010; Zhou *et al*, 2010). The only study that reported an age-dependent effect of ALDH1 expression on prognosis stratified patients by whether they were <65 or >65 years of age (Mieog *et al*, 2012). It is possible that ALDH1 alone is not sufficient for risk stratification among an already high-risk group such as young women. Furthermore, the antibody used in our study is specific for the ALDH1A1 isoform. Although ALDH1A1 is the most commonly evaluated, 16 other isoforms

**Table 3.** Cox regression analysis stratifying by ALDH1 expression (n = 139)

| Parameter    | Odds ratio | s.e. | P-value | 95% Confidence interval |
|--------------|------------|------|---------|-------------------------|
| ER status    | 0.33       | 0.14 | 0.01    | 0.15–0.77               |
| HER2 status  | 1.13       | 0.54 | 0.79    | 0.45–2.88               |
| Tumour grade | 2.67       | 2.95 | 0.38    | 0.30–23.34              |
| Tumour size  | 0.99       | 0.01 | 0.36    | 0.97–1.01               |

Abbreviations: ALDH1 = aldehyde dehydrogenase 1; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor-2.

**Figure 2.** Kaplan and Meier curves of disease-free survival stratifying patients by cytoplasmic ALDH1 expression.

have been identified and the clinical significance of these is uncertain (Sladek, 2003; Marcato *et al*, 2011a, b).

The hypothesis that disease recurrence arises from CSCs that elude conventional chemotherapeutic and irradiation treatments remains an attractive one. However, much remains to be understood about CSCs. One study reported that high ALDH1 expression in tumour-associated stromal cells was associated with a good outcome in triple-negative tumours (De Brot *et al*, 2012). The ALDH1 may have other cellular functions that differ according to cancer type, as seen in ovarian cancer where ALDH1 expression was associated with a favourable outcome instead (Chang *et al*, 2009). Although it is unlikely a coincidence that CSCs are often found to be enriched in poor-risk tumours, it is perhaps oversimplistic to assume that the mere presence of CSCs can account for the more aggressive phenotype and poorer clinical outcome. It is likely that the mechanisms that trigger CSCs re-activation have a greater impact on tumour behaviour and outcome. In fact, CSCs may be the solitary dormant cells in cell cycle arrest (Naumov *et al*, 2002; Allan *et al*, 2006). In this quiescent state, no proliferation occurs and no disease is clinically evident. It remains unclear what triggers an exit from cell cycle arrest into the proliferative phase that leads to tumour regrowth. The multitude of genes other than those involved in stem cell biology that were identified in tumours in younger women suggest that the mechanisms involved are far more complex than can be explained by a single factor such as CSCs (Anders *et al*, 2008).

## CONCLUSION

In conclusion, we observed that ALDH1 expression was more common in younger women and was independently correlated with ER negativity. However, ALDH1 expression did not predict

for disease recurrence or death in these women. Further studies to elucidate the extent of CSC involvement in tumours in younger women and to elucidate the mechanisms that regulate dormancy and cell cycling in CSCs will provide us with a better understanding of tumour biology and facilitate the development of novel therapeutic targets.

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