Somatic copy number changes in *DPYD* are associated with lower risk of recurrence in triple-negative breast cancers

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**Background:** Genomic rearrangements at the fragile site FRA1E may disrupt the dihydropyrimidine dehydrogenase gene (*DPYD*) which is involved in 5-fluorouracil (5-FU) catabolism. In triple-negative breast cancer (TNBC), a subtype of breast cancer frequently deficient in DNA repair, we have investigated the susceptibility to acquire copy number variations (CNVs) in *DPYD* and evaluated their impact on standard adjuvant treatment.

**Methods:** *DPYD* CNVs were analysed in 106 TNBC tumour specimens using multiplex ligation-dependent probe amplification (MLPA) analysis. Dihydropyrimidine dehydrogenase (DPD) expression was determined by immunohistochemistry in 146 tumour tissues.

**Results:** In TNBC, we detected 43 (41%) tumour specimens with genomic deletions and/or duplications within *DPYD* which were associated with higher histological grade (*P* = 0.006) and with rearrangements in the DNA repair gene *BRCA1* (*P* = 0.007). Immunohistochemical analysis revealed low, moderate and high DPD expression in 64%, 29% and 7% of all TNBCs, and in 40%, 53% and 7% of TNBCs with *DPYD* CNVs, respectively. Irrespective of DPD protein levels, the presence of CNVs was significantly related to longer time to progression in patients who had received 5-FU- and/or anthracycline-based polychemotherapy (hazard ratio = 0.26 (95% CI: 0.07–0.91), log-rank *P* = 0.023; adjusted for tumour stage: *P* = 0.037).

**Conclusion:** Genomic rearrangements in *DPYD*, rather than aberrant DPD protein levels, reflect a distinct tumour profile associated with prolonged time to progression upon first-line chemotherapy in TNBC.

Breast cancer is a heterogeneous disease encompassing different subtypes with distinct biological phenotypes and clinical profiles. Among these, triple-negative breast cancer (TNBC) accounts for 15–20% of all breast cancer cases. This subtype is defined by loss of oestrogen- and progesterone receptor (ER and PR) expression as well as lack of human epidermal growth factor receptor-2 (HER2) amplification (Kang *et al*, 2008; Reis-Filho and Tutt, 2008). The majority of TNBCs also exhibit basal-like features. Owing to the absence of specific therapeutic targets such as ER or HER2, adjuvant treatment currently consists of cytotoxic chemotherapy only. Those TNBC patients who do not respond to chemotherapy have an even worse outcome compared with chemoresistant non-TNBC patients (Dent *et al*, 2007; Gluz *et al*, 2009; Linn and Van’t Veer, 2009; Chacon and Costanzo, 2010). A challenging field is,
therefore, the identification of TNBC patients with tumours that are likely to be responsive or resistant to first-line chemotherapy. Individuals with an unfavourable molecular-genetic tumour profile would be potential candidates for alternative treatment modalities exploiting novel molecular targets (Tutt et al, 2010; Fost et al, 2011; Mehta et al, 2011).

Several studies have revealed that a significant number of patients with TNBC respond well to standard treatment with 5-FU and/or anthracyclines (Liedtke et al, 2008; Gluz et al, 2009). More recent studies (Colleoni et al, 2010; Wang et al, 2011) suggested also benefit from the classical CMF (cyclophosphamide, methotrexate, 5-FU) regimen in TNBC. Regarding the efficacy of chemotherapy regimens based on 5-FU or increasingly prescribed 5-FU prodrugs, the key enzyme in the catabolism of (fluoro)pyrimidines is dihydropyrimidine dehydrogenase (DPD). More than 80% of the administered 5-FU is degraded by DPD, thus requiring high standard dosages of the drug (Lu et al, 1993). Low tumoural DPD expression was, therefore, supposed to increase the response rates upon 5-FU treatment (Etienne et al, 1995; Salonga et al, 2000), but the molecular mechanisms leading to altered DPD concentrations in tumour tissues are largely unknown.

In this context, the detection of the common fragile site FRA1E, which extends over 370 kb within the dihydropyrimidine dehydrogenase gene (DPYD) was an important finding, demonstrating a potential mechanism for modifying DPD levels (Hormozian et al, 2007). Common fragile sites are instable chromosomal structures with high DNA torsional flexibility (Schwartz et al, 2006) leading to genomic translocations, amplifications or deletions in case of replication stress. We have recently characterised large intragenic rearrangements within DPYD that occurred in individuals presenting with profound or partial DPD deficiency (van Kuijlenburg et al, 2009; van Kuijlenburg et al, 2010). However, these events revealed to be extremely rare in the germline (Ticha et al, 2009; Pare et al, 2010). As enhanced fragility of common fragile sites has been observed in cancer cells (Arlt et al, 2006), it is conceivable that TNBC tumours which are frequently deficient in BRCA1-mediated DNA repair (Turner and Reis-Filho, 2006; Turner et al, 2007; Rodriguez et al, 2010; Weigman et al, 2012), might be especially susceptible to acquire somatic rearrangements including the DPYD locus. Consequently, disruption of the DPYD gene may have implications for treatment of TNBC with 5-FU containing therapy regimens.

On the basis of these hypotheses, we have evaluated the presence or absence of DPYD copy number variations (CNVs) in TNBC tumour specimens. In this retrospective study, we show that genomic DPYD rearrangements occur frequently and are associated with better patient outcome in TNBC, while mere DPD protein levels did not influence clinical outcome.

PATIENTS AND METHODS

Patients and tumour specimens. One hundred and six fresh-frozen tumour specimens of patients diagnosed with primary TNBC, stored in liquid nitrogen at the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München, were available for studies using high-molecular-weight DNA (cohort 1). Tumour content of the specimens was generally 70% or higher. In addition, nine tissue microarrays (TMAs) were constructed from paraffin-embedded tumour material of 146 TNBC patients, archived at the Institute of Pathology, Technische Universität München, were used for immunohistochemical analyses (cohort 2). In 34 cases, matched fresh-frozen and paraffin-embedded tumour samples were available from the same patient. The patient samples had been collected after surgery between 1988 and 2009 and had been classified and assessed for steroid hormone receptor (ER and PR) and HER2 expression by immunohistochemistry (IHC) (Aubele et al, 2007). Hormone receptor status was defined as negative when less than or equal to 1/2 nuclear staining (Remmele’s score) was observed. Tumours were classified as HER2-negative when assigned as 0 or 1+ by IHC staining and/or lacking of HER2 amplification in FISH staining (according to ASCO guidelines). Samples collected before 1999 were retrospectively assessed for HER2 expression by IHC.

Table 1A. Clinical data and DPYD CNV

| Parameter | n | Valid % | Yes | No | P |
|-----------|---|---------|-----|----|---|
| Cohort 1  | 106 | 41 | 39.8 | 60.2 | 0.718 |
| Age       |    |       |     |    |   |
| < 50      | 62 | 41.9 | 58.1 | 61.7 |
| ≥ 50      | 3  | 41.9 | 58.1 | 61.7 |
| Unknown   | 3  | 41.9 | 58.1 | 61.7 |
| Tumour stage | | | | | |
| pT1 + pT2 | 86 | 84.3 | 15.7 | 12.2 | 0.407 |
| pT3 + pT4 | 16 | 87.8 | 12.2 | 18.3 |
| Unknown   | 4  | 84.3 | 15.7 | 18.3 |
| Nodal status | | | | | |
| N0        | 51 | 52.0 | 48.0 | 41.5 | 0.314 |
| Node-positive | 47 | 58.5 | 41.5 | 51.7 |
| Unknown   | 8  | 48.0 | 52.0 | 51.7 |
| Histological grade | | | | | |
| 1 + 2     | 23 | 23.0 | 9.5  | 32.8 | 0.006* |
| 3         | 77 | 90.5 | 90.5 | 67.2 |
| Unknown   | 6  | 77.0 | 23.0 | 32.8 |
| History   | | | | | |
| Invasive ductal | 82 | 80.9 | 19.1 | 80.0 | 0.732 |
| Medullary  | 6  | 7.1  | 92.9 | 5.0  |
| Other     | 14 | 11.9 | 88.1 | 95.0 |
| Unknown   | 4  | 13.7 | 86.3 | 95.0 |
| BRCA1 CNVs | | | | | |
| Yes       | 63 | 70.0 | 30.0 | 60.3 | 0.007* |
| No        | 27 | 30.0 | 70.0 | 39.7 |
| Unknown   | 16 | 0.0  | 100.0| 61.7 |

| Parameter | n | Valid % | Yes | No | P |
|-----------|---|---------|-----|----|---|
| chemotherapy | | | | | |
| None      | 19 | 19.4 | 14.6 | 22.8 |
| FEC       | 27 | 27.6 | 29.3 | 62.6 |
| CMF       | 16 | 16.3 | 22.0 | 12.3 |
| EC-CMF    | 4  | 4.1  | 95.9 | 87.7 |
| EC        | 21 | 21.4 | 78.6 | 12.3 |
| Other     | 11 | 11.2 | 88.8 | 12.3 |
| Unknown   | 8  | 9.7  | 90.3 | 12.3 |

Abbreviations: CMF = cyclophosphamide, methotrexate, 5-FU; CNVs = copy number variations; DPYD = dihydropyrimidine dehydrogenase; EC = epirubicin, cyclophosphamide; FEC, 5-FU = epirubicin, cyclophosphamide.

*Statistically significant.
Clinical parameters of the studied patient panels are listed in Tables 1A and 1B. Approximately 80% of the tumour specimens were classified as invasive ductal carcinoma of no special type and were categorised in the high-grade group (G3) according to the classification of Elston and Ellis (Elston and Ellis, 1991). Patients had received treatment following the guidelines at the time of diagnosis. For patient cohort 1, follow-up data were recorded for up to 203 months and were available for 91 patients including 69 patients treated with standard 5-FU- and/or anthracycline-based polychemotherapy and 65 patients who had received radiotherapy. The 5-year overall survival (OS) and progression-free survival (time to progression (TTP)) rates in patients treated with polychemotherapy were 81 ± 5.1% (standard error SE) and 76 ± 5.5%, respectively. For patient cohort 2, 105 cases with follow-up data up to 244 months were available. Sixty-eight of these patients had received 5-FU- and/or anthracycline-based polychemotherapy and showed 5-year OS and progression-free survival (TTP) rates of 80 ± 5.1% and 65 ± 6.1%, respectively. Patients with neoadjuvant treatment were excluded from survival calculations. Written informed consent for the use of tissue samples for research purposes was obtained from all patients. Approval for use of the tumour samples was received from the Ethics Committee of the Medical Faculty of the Technische Universität München.

DNA preparation. Nuclear fractions were prepared from frozen TNBC specimens after routine separation of cytosol preparations by ultracentrifugation (Janicke et al, 1994). High-molecular weight DNA was extracted from nuclear fractions by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Five blood samples of healthy donors were prepared with the same kit and used as control samples for multiplex ligation-dependent probe amplification (MLPA) analysis.

MLPA analysis. The MLPA test for DPYD (P103-B1, MRC-Holland, Amsterdam, The Netherlands) is composed of 38 probes for DPYD, including one probe for detecting the c.1905 + 1G > A mutation, and nine control probes specific for DNA sequences outside the DPYD gene. The MLPA test was performed as described before (Schouten et al, 2002; van Kuilenburg et al, 2010) using 50–200 ng of DNA per reaction. Data analysis was performed using GeneMarker software (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). The relative peak area was determined by dividing the mean of the peak areas of the control probes of each sample by the mean of the peak areas of the control probes of all the samples \( R_{\text{control}} \). The peak area of each DPYD MLPA probe of a sample was divided by the \( R_{\text{control}} \) of that sample. Subsequently, the relative peak area of each DPYD probe was divided by the average relative peak area of this probe in all the tumour samples. In unaffected individuals, this will result in a value of 1 (100%) representing two copies of the target sequence in the sample. According to the manufacturer’s recommendations, we applied cut-off values for each probe ratio of <0.70 and >1.30, respectively, to define reduced or increased copy numbers of the target sequence. As the MLPA test enables detection of an aberration in a region covered by multiple MLPA probes if 20% or even less aberrant tumour cells are present (Hömg-Hölzel and Savola, 2012), we additionally included samples with a mean probe ratio of all DPYD probes below \( \leq 0.85 \) or above \( \geq 1.15 \), indicative for a cell fraction with deletion or duplication of the whole coding region. Samples were analysed in duplicate runs and five blood samples were included as reference.

The MLPA test for BRCA1 (P002-C1, MRC-Holland, Amsterdam, The Netherlands) contains 26 probes for BRCA1 and 9 control probes specific for DNA sequences outside the BRCA1 gene. The relative peak areas were determined as described above. Subsequently, the relative peak area of each BRCA1 probe was divided by the average relative peak area of this probe obtained from five blood samples.

Generation of anti-DPD antiserum. Human DPYD cDNA was cloned into the Nco I/Bgl II restriction sites of a pQE-60 vector (Qiagen) including a polyhistidine (His_6)-tag at the C-terminus. The recombinant protein was expressed in Escherichia coli cells and subsequently purified by nickel-nitritrotriacetic acid sepharose chromatography (Qiagen) according to standard procedures. Following dialysis and re-naturation in phosphate-buffered saline, 1 ml DTt, pH 7.4, two rabbits were immunised with this protein preparation. Anti-DPD antibodies were affinity-purified by coupling the immunogen preparation to a mixture of 50%/50% AffiGel-10 and AffiGel-15 (BioRad, München, Germany). Elution was performed with 0.1 M glycine/HCl buffer pH 2.4, followed by re-neutralisation to pH 7.4. Finally, antibodies were concentrated by ultrafiltration with Ultracel 50 K (Merck Millipore, Schwalbach, Germany).
Germany) and diluted 50%/50% (v/v) with glycerol for storage. All batches were checked by one-site ELISA using DPD coated on microplates. Immunoreactivity with the specific DPD band at \(\sim 105\,\text{kDa}\) was confirmed by western blot using protein preparations obtained from \(E.\ coli\) cells expressing the recombinant protein as well as from peripheral blood mononuclear cells.

**Immunohistochemistry.** DPD protein expression was measured by IHC using TMAs (Aubele et al., 2007). Tissue microarray sections were deparaffinized and rehydrated through a graded ethanol series finishing with distilled water. Endogenous peroxidase was inhibited by treatment with 3% hydrogen peroxide. The affinity-purified rabbit antibody anti-DPD antiserum was applied in a dilution of 1:300 and incubated for 1 h at room temperature in humidified chambers. Staining was performed with the Dako EnVision Detection System (Dako, Hamburg, Germany) which uses a peroxidase-conjugated polymer backbone coupled to secondary antibody molecules, and diaminobenzidine (DAB +) as chromogenic substrate. Nuclei of the cells were finally counterstained with haematoxylin. Dihydropyrimidine dehydrogenase staining intensity was assigned as absent (0), low (1), or strong (3) staining (Figure 2A–F). Normal ductal epithelium (panel A) or intraductal carcinoma exhibited strong (3+) staining. Compared with ductal epithelia, the majority of TNBC (64%) as well as TPBC (90%) tumour specimens showed profound downregulation of DPD protein levels (score 0–1+). Moreover, we observed lower DPD expression in higher stage tumours (pT3 + pT4 categories) exhibiting statistical significance (\(P = 0.003;\) Table 1B and 2).

To evaluate the DPD expression status in TNBCs with or without \(DPYD\) CNVs, we defined the staining pattern in 34 matched cases for which both IHC and MLPA data were available (Table 2). Loss of heterozygosity in the \(DPYD\) gene was indeed accompanied by low (0–1+) to moderate (2+) DPD staining in all except one tumour sample. Similarly, cancers exhibiting duplications were predominately (67%) associated with moderate (2+) DPD staining. One TNBC sample with a complex pattern of deleted and amplified sequences showed total loss of DPD protein (see also Figure 2B and E). Nevertheless, DPD expression scores and MLPA data did not reveal a statistical correlation (\(P = 0.461\)) as tumour specimens without any \(DPYD\) rearrangements showed a broad spectrum of DPD staining as well ranging from undetectable up to high expression levels (Table 2).

As low DPD expression has been suggested to increase the response rates in cancer patients treated with the fluoropyrimidine drug 5-FU, we analysed outcome in TNBC patients who had received adjuvant treatment. However, even in the subgroup of patients treated with 5-FU-based chemotherapy only \((n = 46)\), we did not observe a clinical benefit from low (0–1+) DPD protein expression compared with moderate and high expression for TTP (5-year TTP with low DPD expression: 65.5 ± 8.8%; moderate expression: 82.5 ± 11.3%; strong expression: 75.0 ± 21.7%; log-rank \(P = 0.383\)).

**DPYD CNVs have prognostic value and are associated with longer TTP.** We next assessed the impact of \(DPYD\) CNVs (applied as combined status of deletions or duplications) on TTP and OS (Table 3). In the patient subgroup treated with standard adjuvant polychemotherapy (cohort 1), 32 of 69 TNBCs showed genomic \(DPYD\) rearrangements which were significantly associated with a reduced risk of disease progression (HR = 0.26 [95% CI: 0.07–0.91], log-rank \(P = 0.023\)) (Figure 3A). Tumour stage revealed to be the only prognostic clinical parameter for TTP in the univariate analysis (Table 3). Adjusted for tumour stage, the \(DPYD\) status remained to be an independent parameter providing additional prognostic information (\(P = 0.037\)). Moreover, in the multivariable model including the most important established clinical factors (age, histological grading, nodal status and tumour stage) (Table 4A), \(DPYD\) CNVs and tumour stage maintained statistical significance as well although the small number of events \((n = 16)\) may weaken the power of this model. The \(DPYD\) status did not provide prognostic information independent of the \(BRCA1\)
status (Table 4B) underlining the observed association of DPYD and BRCA1 CNVs.

The TTP was also assessed in the subgroup of all patients who had received 5-FU (n = 45). In patients with DPYD CNVs, higher 5-year progression-free survival rates were observed compared with patients exhibiting normal copy numbers (86 ± 7.3% and 64 ± 10.2%, respectively), however, statistical significance was not reached for overall comparison (log-rank P = 0.074) (Figure 3B).

In patients who had received radiotherapy (n = 65), we also observed a tendency towards prolonged TTP with DPYD CNVs (log-rank P = 0.056). Five-year progression-free survival rates were 87.5 ± 6.8% in patients with DPYD CNVs compared with 64 ± 8.1% in patients with no CNVs (Figure 3C).

**DISCUSSION**

Intra-tumoural levels of DPD have been suggested to be an important prognostic factor for the efficacy of 5-FU-based chemotherapy regimens (Etienne et al, 1995; Salonga et al, 2000). For breast cancer, Horiguchi et al reported better patient outcome in case of low DPD concentrations (Horiguchi et al, 2002). Therefore, it was our intention to characterise the DPD/DPYD status in TNBC, a breast cancer subtype which is frequently treated with 5-FU-containing polychemotherapy. Here we show that DPD protein expression is profoundly downregulated (compared with normal ductal epithelium) in 64% of the TNBC specimens which is...
consistent with previous data obtained in other neoplastic cells (Holstege et al., 1986; Shiotani et al., 1989a,b).

The mechanistic basis of DPD downregulation in cancer is largely unknown so far. Among breast cancers, the triple-negative/basal-like subtype is supposed to exhibit the greatest degree of genomic instability showing common loss of important DNA repair genes (Weigman et al., 2012). Thus, we hypothesised that this phenotype might favour recombination events including the fragile site FRA1E which disrupts the DPYD gene (Hormozian et al., 2007). Indeed, we observed for the first time a high prevalence of 41% of genomic DPYD rearrangements in TNBC tumour specimens, whereas their population incidence in the germline is extremely rare (Ticha et al., 2009; Pare et al., 2010). Secondly, our data revealed that CNVs within DPYD were positively associated with aberrant copy numbers of BRCA1. This is in line with the copy number studies by Weigman et al. (2012) who suggested loss of BRCA1-dependent DNA repair might be involved in overall

| Variable | TTP (n = 69) | OS (n = 73) |
|----------|-------------|-------------|
| Age | | |
| < 50 | 1.0 | 1.0 |
| > 50 | 2.31 | 0.80–6.67 | 0.109 |
| | 4.01 | 1.12–14.40 | 0.021* |
| Tumour stage | | |
| pT1 + 2 | 4.38 | 1.24–15.43 | 0.012* |
| pT3 + 4 | | 1.0 | 1.0 |
| | 5.25 | 1.45–18.99 | 0.005* |
| Nodal status | | |
| N0 | 1.60 | 0.60–4.31 | 0.344 |
| Node-positive | 1.92 | 0.63–5.86 | 0.245 |
| Histological grade | | |
| 1 + 2 | 1.0 | 0.91 | 0.26–3.21 | 0.886 |
| 3 | | 1.0 | 1.0 |
| | 2.62 | 0.34–20.05 | 0.335 |
| DPYD status | | |
| No CNV | 1.0 | | 1.0 |
| CNV | 0.26 | 0.07–0.91 | 0.023* |
| BRCA1 status | | |
| No CNV | 1.0 | | 1.0 |
| CNV | 0.36 | 0.11–1.14 | 0.069 |

Abbreviations: CNV = copy number variation; DPYD = dihydropyrimidine dehydrogenase gene; IHC = immunohistochemistry; TNBC = triple-negative breast cancer; TPBC = triple-positive breast cancer (hormone receptor-positive and HER2-positive).

a Matched cases of TNBC specimens examined for DPD expression and DPYD CNV.

b Correlation of copy number data with low versus moderate/high protein scores: P = 0.461 (Fisher’s exact test).

c Two out of four cases consisted of duplications of the entire coding region.

Table 3. Effect of clinical and molecular-genetic parameters on outcome of patients treated with 5-FU and/or anthracycline-based therapy

Table 2. DPD protein expression scores determined by IHC

| DPD expression score (IHC) | n | Low (0–1+) | Moderate (2+) | High (3+) |
|---------------------------|---|------------|--------------|-----------|
| TNBC (all) | 146 | 93 (64) | 42 (29) | 11 (7) |
| TNBCs with DPYD CNVs | | | | |
| Deletion | 9 | 4 (44) | 4 (44) | 1 (11) |
| Duplication | 6 | 2 (33) | 4 (67)* | 0 |
| No CNV | 19 | 12 (63) | 6 (32) | 1 (5) |
| Comparative tissue | | | | |
| TPBC | 20 | 18 (90) | 2 (10) |

Abbreviations: CNV = copy number variation; DPYD = dihydropyrimidine dehydrogenase gene; IHC = immunoistochemistry; TNBC = triple-negative breast cancer; TPBC = triple-positive breast cancer (hormone receptor-positive and HER2-positive).

Figure 2. DPD protein expression assessed by immunohistochemical staining of TNBC specimens with anti-DPD. DPD expression was measured by immunohistochemistry using an affinity-purified anti-DPD antibody. Tissue microarray sections with different staining intensity are shown. (A) Section showing normal ductal epithelium with strong (3+) DPD staining. (B, C) Invasive breast cancer of no special type (NST) with undetectable DPD expression; parallel analysis of the DPYD gene in (B) revealed large deleted and amplified regions within the coding sequence. (D) Invasive breast cancer, NST, with low (1+) DPD expression. (E) Invasive breast cancer, NST, showing moderate (2+) DPD expression of tumour cells; parallel analysis of the DPYD gene suggested the presence of a duplication (mean copy number = 150% of normal control). (F) Invasive breast cancer, NST, showing strong DPD expression assigned as 3+. Abbreviations: ND = normal ductal epithelium; ST = stroma; TU = tumour cells.
Five-year TTP rates for patients with DPYD CNVs and wildtype to the presence of DPYD DPD expression in tumour tissues with wildtype copy). Accordingly, we mainly found moderate to low tumour specimen and/or the presence of a remaining majority of samples with to inactivate the gene as they occurred within the coding sequence. M checkpoint function (Arlt et al, 2012) is required for the stability of common fragile sites through its G2/M checkpoint function (Arlt et al, 2012) and, thus, suggest increased vulnerability of these TNBC tumours towards DNA-damaging agents such as radiation, DNA-intercalating anthracyclines or alkylating cyclophosphamide. Also, benefit from newly developed agents, for example, PARP inhibitors (Tutt et al, 2010), targeting DNA-repair-deficient cancers, might be expected. As the prognostic value of molecular signatures of the first generation was

accessible.

Table 4A. Multivariable analysis of risk of progression

| Variable                  | HR*   | 95% CI         | P    |
|---------------------------|-------|----------------|------|
| DPYD status: No CNV vs CNV| 0.27  | 0.075–0.96     | 0.048* |
| Age: <50 years vs >50 years| 2.63  | 0.87–7.92      | 0.087 |
| Grading: G1 + G2 vs G3    | 1.16  | 0.31–4.36      | 0.822 |
| Tumour stage: pT 1 + 2 vs 3 + 4 | 4.71  | 1.06–20.95     | 0.042* |
| Nodal status: none vs positive | 1.27  | 0.42–3.83      | 0.672 |

Abbreviations: CNV = copy number variation; HR = hazard ratio. Total number of patients in analysis: 67, number of events: 16.

* Cox proportional HR for risk of progression in patients treated with adjuvant polychemotherapy containing 5-FU and/or anthracyclines.

**Statistically significant.

Table 4B. DPYD CNVs adjusted for BRCA1 CNVs

| Variable                  | HR*   | 95% CI         | P   |
|---------------------------|-------|----------------|-----|
| DPYD status: No CNV vs CNV| 0.37  | 0.078–1.71     | 0.201 |
| BRCA1 status: No CNV vs CNV| 0.43  | 0.133–1.37     | 0.151 |

Abbreviations: CI = confidence interval; CNV = copy number variation; HR = hazard ratio. Total number of patients in analysis: 57, number of events: 12.

* Cox proportional HR for risk of progression in patients treated with adjuvant polychemotherapy containing 5-FU and/or anthracyclines.

Figure 3. Kaplan–Meier curves demonstrating the effect of DPYD status on time to progression (TTP). (A) TNBC patients treated with standard 5-FU- and/or anthracycline-based chemotherapy (n = 69). Somatic copy number changes (CNVs) in DPYD were significantly associated with longer TTP compared with DPYD-wildtype TNBC tissues. Five-year TTP rates for patients with aberrant DPYD and wildtype DPYD were estimated to be 90 ± 5.5% and 65.5 ± 8.2%, respectively. (B) TNBC subset treated with 5-FU-containing chemotherapy (n = 45). Five-year TTP rates for patients with DPYD CNVs and wildtype DPYD were estimated to be 86 ± 7.3% and 64 ± 10.2%, respectively. (C) TNBC subset treated with radiotherapy (n = 65). Five-year TTP rates for patients with DPYD CNVs and wildtype DPYD were estimated to be 87.5 ± 6.8% and 64 ± 8.1%, respectively.

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| DPYD status: No CNV vs CNV| 0.37  | 0.078–1.71     | 0.201 |
| BRCA1 status: No CNV vs CNV| 0.43  | 0.133–1.37     | 0.151 |

Abbreviations: CI = confidence interval; CNV = copy number variation; HR = hazard ratio. Total number of patients in analysis: 57, number of events: 12.

* Cox proportional HR for risk of progression in patients treated with adjuvant polychemotherapy containing 5-FU and/or anthracyclines.

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Figure 3. Kaplan–Meier curves demonstrating the effect of DPYD status on time to progression (TTP). (A) TNBC patients treated with standard 5-FU- and/or anthracycline-based chemotherapy (n = 69). Somatic copy number changes (CNVs) in DPYD were significantly associated with longer TTP compared with DPYD-wildtype TNBC tissues. Five-year TTP rates for patients with aberrant DPYD and wildtype DPYD were estimated to be 90 ± 5.5% and 65.5 ± 8.2%, respectively. (B) TNBC subset treated with 5-FU-containing chemotherapy (n = 45). Five-year TTP rates for patients with DPYD CNVs and wildtype DPYD were estimated to be 86 ± 7.3% and 64 ± 10.2%, respectively. (C) TNBC subset treated with radiotherapy (n = 65). Five-year TTP rates for patients with DPYD CNVs and wildtype DPYD were estimated to be 87.5 ± 6.8% and 64 ± 8.1%, respectively.

Table 4A. Multivariable analysis of risk of progression

| Variable                  | HR*   | 95% CI         | P    |
|---------------------------|-------|----------------|------|
| DPYD status: No CNV vs CNV| 0.27  | 0.075–0.96     | 0.048* |
| Age: <50 years vs >50 years| 2.63  | 0.87–7.92      | 0.087 |
| Grading: G1 + G2 vs G3    | 1.16  | 0.31–4.36      | 0.822 |
| Tumour stage: pT 1 + 2 vs 3 + 4 | 4.71  | 1.06–20.95     | 0.042* |
| Nodal status: none vs positive | 1.27  | 0.42–3.83      | 0.672 |

Abbreviations: CNV = copy number variation; HR = hazard ratio. Total number of patients in analysis: 67, number of events: 16.

* Cox proportional HR for risk of progression in patients treated with adjuvant polychemotherapy containing 5-FU and/or anthracyclines.

**Statistically significant.

Table 4B. DPYD CNVs adjusted for BRCA1 CNVs

| Variable                  | HR*   | 95% CI         | P   |
|---------------------------|-------|----------------|-----|
| DPYD status: No CNV vs CNV| 0.37  | 0.078–1.71     | 0.201 |
| BRCA1 status: No CNV vs CNV| 0.43  | 0.133–1.37     | 0.151 |

Abbreviations: CI = confidence interval; CNV = copy number variation; HR = hazard ratio. Total number of patients in analysis: 57, number of events: 12.

* Cox proportional HR for risk of progression in patients treated with adjuvant polychemotherapy containing 5-FU and/or anthracyclines.
shown to be related to ER-positive, rather than ER-negative breast cancers (Reis-Filho and Pusztai, 2011), there is still a need for prognostic or predictive factors for the TNBC subgroup. Genomic DPYD rearrangements might thus arise as a candidate marker suitable for validation in larger clinical studies.

In conclusion, we detected a high prevalence of somatic copy number aberrations in TNBCs affecting the DPYD gene. The presence of DPYD CNVs might help to subdivide TNBCs into molecular classes with better prognosis, while low DPD protein expression in general had no impact on better patient outcome. Patients with aberrant DPYD copy numbers might therefore be well suited for treatment with standard polychemotherapy combined with radiotherapy. Further studies will show whether genomic DPYD rearrangements might be incorporated into a panel of novel molecular signatures predicting clinical outcome in TNBC.

ACKNOWLEDGEMENTS

We are grateful to Fred Sweep, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, for his support in the generation of anti-DPD antibodies. We thank Daniela Hellmann for excellent technical assistance. This work was partly financed by Wilhelm Sander-Stiftung, Munich, Germany, contract number 2012.028.1.

CONFLICT OF INTEREST

R. Vijzelaar is employed by MRC-Holland b.v. which supplies the MLA kits. All remaining authors have declared no conflict of interest.

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