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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,

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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 oral 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 Kuilenburg *et al*, 2009; van Kuilenburg *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) which 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 3/12 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 %	DPYD CNVs (% patients)		P
			Yes	No	
Cohort 1	106		n=43	n=63	
Age					
<50	41	39.8	41.9	38.3	0.718
≥50	62	60.2	58.1	61.7	
Unknown	3				
Tumour stage					
pT1 + pT2	86	84.3	87.8	81.7	0.407
pT3 + pT4	16	15.7	12.2	18.3	
Unknown	4				
Nodal status					
N0	51	52.0	58.5	48.3	0.314
Node-positive	47	48.0	41.5	51.7	
Unknown	8				
Histological grade					
1 + 2	23	23.0	9.5	32.8	0.006 ^a
3	77	77.0	90.5	67.2	
Unknown	6				
Histology					
Invasive ductal	82	80.4	80.9	80.0	0.732
Medullary	6	5.9	7.1	5.0	
Other	14	13.7	11.9	15.0	
Unknown	4				
BRCA1 CNVs					
Yes	63	70.0	87.5	60.3	0.007 ^a
No	27	30.0	12.5	39.7	
Unknown	16				
Chemotherapy					
None	19	19.4	14.6	22.8	0.714
FEC	27	27.6	29.3	26.3	
CMF	16	16.3	22.0	12.3	
EC-CMF	4	4.1	4.8	3.5	
EC	21	21.4	19.5	22.8	
Other	11	11.2	9.7	12.3	
Unknown	8				
Abbreviations: CMF = cyclophosphamide, methotrexate, 5-FU; CNVs = copy number variations; DPYD = dihydropyrimidine dehydrogenase; EC = epirubicin, cyclophosphamide; FEC, 5-FU = epirubicin, cyclophosphamide.					
^a Statistically significant.					
DPYD CNVs included 21 deletions and 19 duplications (see text). BRCA1 CNVs included 60 deletions, two intragenic duplications and one gene duplication associated with a mutation.					

Table 1B. Clinical data and DPD protein expression (IHC)

Parameter	n	Valid %	DPD expression score (% patients)		P
			0-1 +	2-3 +	
Cohort 2	146		n = 93	n = 53	
Age					
< 50	39	28.7	25.0	35.4	0.199
≥ 50	97	71.3	75.0	64.6	
Unknown	10				
Tumour stage					
pT1 + pT2	107	76.4	68.2	90.4	0.003 ^a
pT3 + pT4	33	23.6	31.8	9.6	
Unknown	6				
Nodal status					
N0	59	45.4	45.2	45.7	0.964
Node-positive	71	54.6	54.8	54.3	
Unknown	16				
Histological grade					
1 + 2	17	12.7	14.3	10.0	0.471
3	117	87.3	85.7	90.0	
Unknown	12				
Histology					
Invasive ductal	115	80.4	76.7	86.8	0.277
Medullary	18	12.6	13.3	11.3	
Other	10	7.0	10.0	1.9	
Unknown	3				
Chemotherapy					
None	54	37.5	42.9	28.3	0.744
FEC	26	18.1	16.5	20.8	
CMF	21	14.6	14.3	15.1	
EC-CMF	7	4.9	4.4	5.7	
EC	24	16.7	15.4	18.9	
Other	12	8.4	6.6	11.4	
Unknown	2				
Abbreviations: CMF = cyclophosphamide, methotrexate, 5-FU; DPYD = dihydropyrimidine dehydrogenase; EC = epirubicin, cyclophosphamide; FEC = 5-FU, epirubicin, cyclophosphamide; IHC = immunohistochemistry.					
^a 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 $81 \pm 4.7\%$ (standard error SE) and $76 \pm 5.5\%$, 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 $80 \pm 5.1\%$ and $65 \pm 6.1\%$, 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 GeneMapper 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_{control}). The peak area of each *DPYD* MLPA probe of a sample was divided by the R_{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 (Hömig-Hölzel and Savola, 2012), we additionally included samples with a mean probe ratio of all *DPYD* probes below ≤ 0.85 or above ≥ 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-nitrilotriacetic acid sepharose chromatography (Qiagen) according to standard procedures. Following dialysis and re-naturation in phosphate-buffered saline, 1 mM 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 Ultracell 50 K (Merck Millipore, Schwalbach,

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 ~105 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 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+), moderate (2+) or strong (3+) staining. To confirm the adequacy of the immunohistochemical staining, mammary ductal epithelium (Kamoshida *et al*, 2005) and liver tissue (Ho *et al*, 1986; Gerlach *et al*, 2011) were used as positive controls known to exhibit high amounts of DPD protein or mRNA. Furthermore, an additional breast cancer tissue section was included in each run as negative control by omission of primary antibody (Punsawad *et al*, 2013).

Statistics. Data independently obtained from MLPA and IHC analyses were merged at the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München. Statistical analysis was performed with the IBM SPSS Statistics software version 19.0 (SPSS Inc., Chicago, IL, USA). The OS and TTP were considered as long-term endpoints. The OS was defined as the time from surgery until death from any cause and TTP was defined as the time from surgery to the first incidence of disease recurrence (local or distant). The Cox proportional hazard model was used to assess univariate and multivariable explanatory ability of the clinical or molecular parameters with respect to OS and TTP. Survival curves were generated according to the Kaplan–Meier method and the log-rank test was used for statistical comparison of event-time distributions between independent subgroups. Ninety-five per cent confidence intervals were provided for relevant effect estimates such as hazard ratios (HRs). Association of molecular and categorical clinical data was assessed by the Chi-square test. All statistical tests were conducted two-sided and a *P*-value <0.05 was considered to indicate statistical significance. To retain a maximum of power in the primary interesting analyses, no correction of *P*-values was applied to adjust for multiple testing (Saville, 1990). This study was designed following the reporting recommendations for tumour marker prognostic studies (REMARK) (McShane *et al*, 2006; Altman *et al*, 2012).

RESULTS

DPYD CNVs occur frequently in TNBC tumour specimens. One hundred and six fresh-frozen TNBC specimens (cohort 1) were analysed by MLPA to investigate the prevalence of large rearrangements within the *DPYD* gene. We detected CNVs of *DPYD* exons in 43 tumour specimens (41%, 95% CI: 31–51%). Eleven samples exhibited breakpoints within the FRA1E block spanning *DPYD* exons 13–16, a region supposed to display highest recombination frequency (Hormozian *et al*, 2007). Overall, however, gains or losses were observed throughout the entire gene without apparent hotspots. Six of 19 duplications and 11 of 21

deletions extended over the whole *DPYD* coding sequence as exemplarily illustrated in Figure 1. Furthermore, three TNBC samples showed a more complex pattern with large deleted as well as amplified regions within the gene.

As expected, *DPYD* CNVs were predominantly found in the group of high-grade (G3) tumours (Table 1A; *P*=0.006). Interestingly, we also observed a significant association of *DPYD* CNVs with the incidence of reduced/aberrant copies of *BRCA1* (*P*=0.007). Age, tumour stage or nodal status (binary variables) were not significantly linked to *DPYD* CNVs (Table 1A).

DPD protein expression is frequently downregulated in TNBC tumour specimens. We assessed the DPD expression status in TNBC tissues (cohort 2) using IHC. For this purpose, nine TMAs containing tumour tissue sections of 146 triple-negative and 20 triple-positive breast cancer (TPBC) patients were constructed. A broad range of cytoplasmic staining with the anti-DPD antibody was observed ranging from undetectable (0) to 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 Table 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 predominantly (67%) associated with moderate (2+) DPD staining, 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*

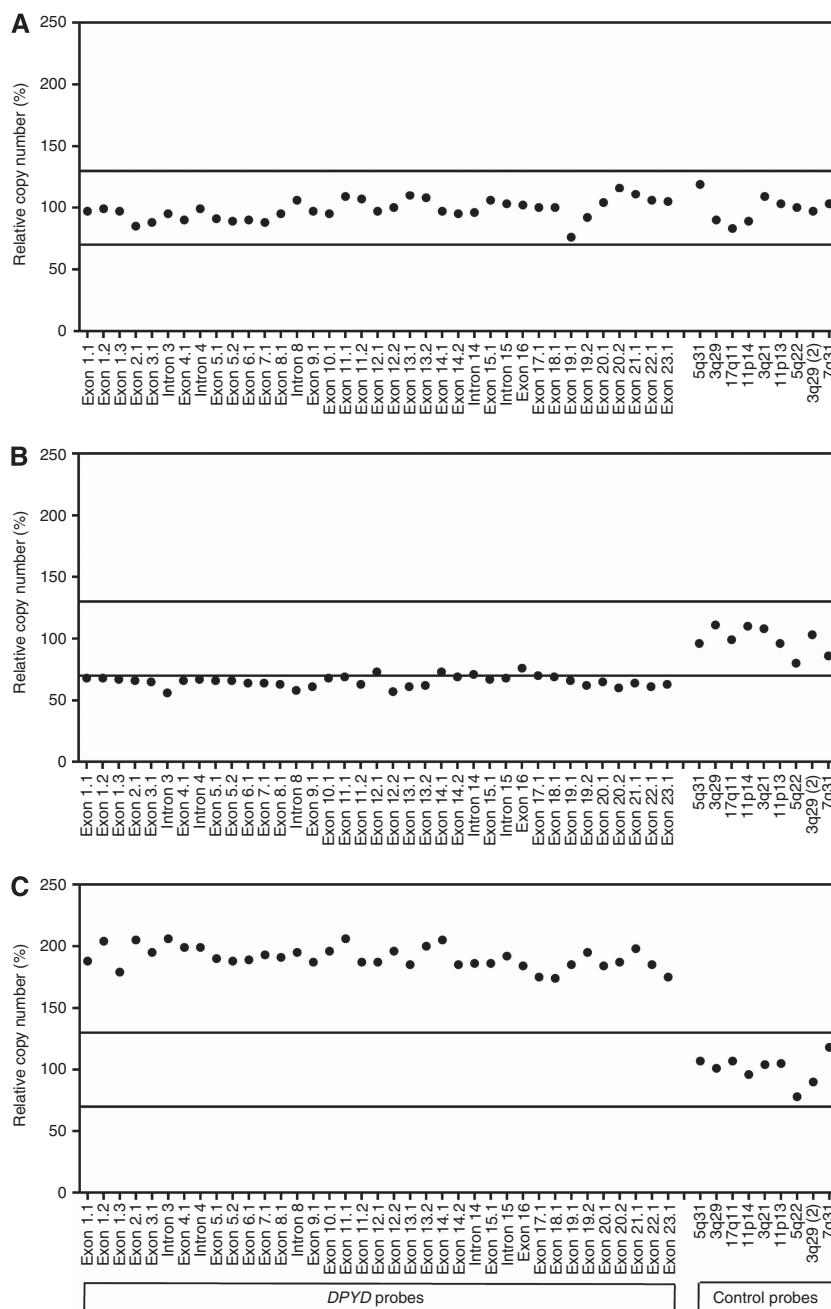


Figure 1. Analysis of copy number changes in *DPYD* using MLPA. The results of the quantitative analysis of the copy number of the 23 coding exons and 4 intronic sequences of *DPYD* and 9 control probes specific for DNA sequences outside *DPYD* is shown for a patient with no aberrations (panel A), deletion of the entire *DPYD* gene (panel B) and amplification of the entire *DPYD* gene (panel C). The solid lines represent the cut-off values indicative for amplification (relative copy number > 1.3) or deletion (relative copy number < 0.7) of that particular sequence.

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 ($86 \pm 7.3\%$ and $64 \pm 10.2\%$, 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 $87.5 \pm 6.8\%$ in patients with *DPYD* CNVs compared with $64 \pm 8.1\%$ 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

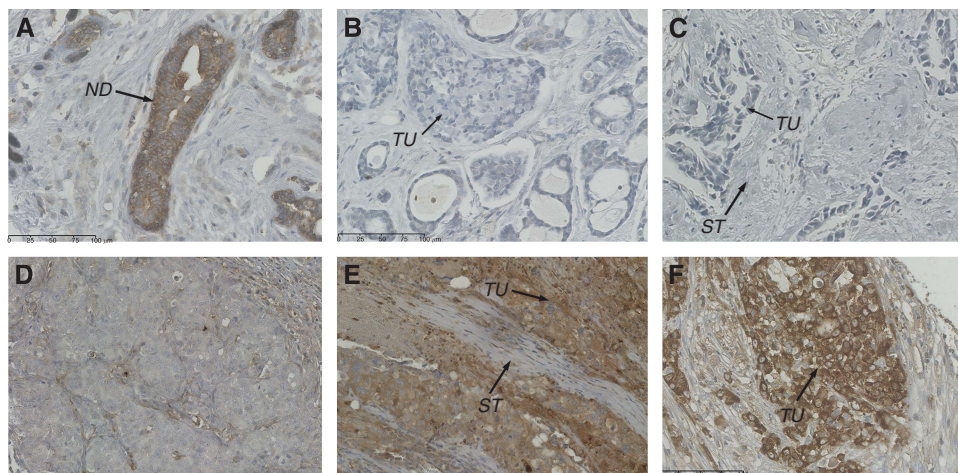


Figure 2. DPD protein expression assessed by immunohistochemical staining of TNBC specimens with anti-DPD. DPD expression was measured by immunohistochemistry using an affinity-purified anti-DPD antibody. Tissue microarray sections with different staining intensity are shown. (A) Section showing normal 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.

	n	DPD expression score (IHC)		
		Low (0-1+) n (%)	Moderate (2+) n (%)	High (3+) n (%)
TNBC (all)	146	93 (64)	42 (29)	11 (7)
TNBCs with DPYD CNVs^{a,b}				
Deletion	9	4 (44)	4 (44)	1 (11)
Duplication	6	2 (33)	4 (67) ^c	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 = immunohistochemistry; TNBC = triple-negative breast cancer; TPBC = triple-positive breast cancer (hormone receptor-positive and HER2-positive).
^aMatched cases of TNBC specimens examined for DPD expression and DPYD CNV.
^bCorrelation of copy number data with low versus moderate/high protein scores: $P = 0.461$ (Fisher's exact test).
^cTwo out of four cases consisted of duplications of the entire coding region.

Variable	TTP (n = 69)			OS (n = 73)		
	HR	95% CI	Log-rank p	HR	95% CI	Log-rank p
Age						
<50	1.0			1.0		
≥50	2.31	0.80-6.67	0.109	4.01	1.12-14.40	0.021 ^a
Tumour stage						
pT1+2	1.0			1.0		
pT3+4	4.38	1.24-15.43	0.012 ^a	5.25	1.45-18.99	0.005 ^a
Nodal status						
N0	1.0			1.0		
Node-positive	1.60	0.60-4.31	0.344	1.92	0.63-5.86	0.245
Histological grade						
1+2	1.0			1.0		
3	0.91	0.26-3.21	0.886	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 ^a	0.89	0.31-2.56	0.831
BRCA1 status						
No CNV	1.0			1.0		
CNV	0.36	0.11-1.14	0.069	1.27	0.27-5.87	0.761

Abbreviations: 95% CI = 95% confidence interval; CNV = copy number variation; HR = hazard ratio; OS = overall survival; TTP = time to progression.
^aStatistically significant.

consistent with previous data obtained in other neoplastic cells (Holsteg *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

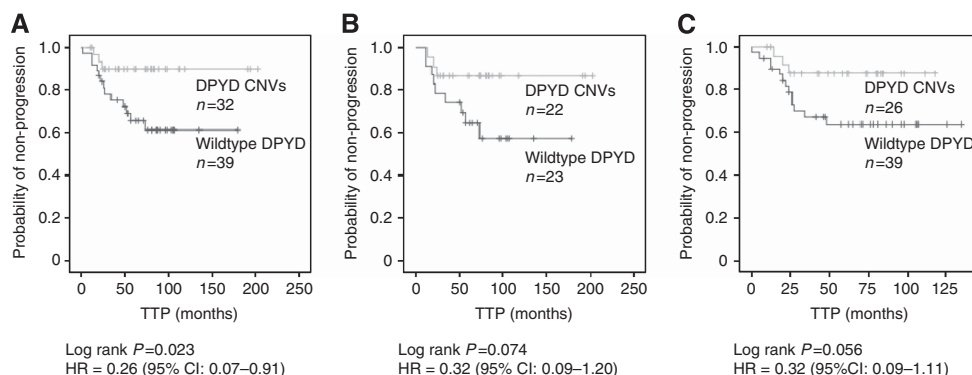


Figure 3. Kaplan–Meier curves demonstrating the effect of *DPYD* status on time to progression (TTP). (A) TNBC patients treated with standard 5-FU- and/or anthracycline-based chemotherapy ($n = 69$). Somatic copy number changes (CNVs) in *DPYD* were significantly associated with longer TTP compared with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

Table 4A. Multivariable analysis of risk of progression

Variable	HR ^a	95% CI	P
<i>DPYD</i> status: No CNV vs CNV	0.27	0.075–0.96	0.043^b
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^b
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.

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

^bstatistically significant

Table 4B. *DPYD* CNVs adjusted for *BRCA1* CNVs

Variable	HR ^a	95% CI	P
<i>DPYD</i> status: No CNV vs CNV	0.37	0.078–1.71	0.201
<i>BRCA1</i> 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.

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

genomic instability in basal-like/TNBCs. Furthermore, our observations may reflect greater vulnerability of the FRA1E site in the background of *BRCA1* abnormalities, as intact *BRCA1* appears to be required for the stability of common fragile sites through its G2/M checkpoint function (Arlt *et al*, 2004).

Most of the 43 aberrations we had observed in *DPYD* were likely to inactivate the gene as they occurred within the coding sequence. Only six TNBCs exhibited entire gene duplications. Thus, the majority of samples with *DPYD* CNVs should be associated with a more or less pronounced decrease of transcript and protein levels (depending on the percentage of cells with aberrant *DPYD* in the tumour specimen and/or the presence of a remaining *DPYD* wildtype copy). Accordingly, we mainly found moderate to low DPD expression in tumour tissues with *DPYD* rearrangements. On the other hand, low DPD protein expression was not restricted to the presence of *DPYD* CNVs, but was also evident in the

majority (>60%) of tumours without any aberrations. Hence, additional mechanisms for *DPYD* downregulation, for example, epigenetic or transcriptional regulations (Ezzeldin *et al*, 2005; Zhang *et al*, 2006) are likely to occur. Taken together, the presence of *DPYD* CNVs is only one parameter among others which altogether may influence DPD protein levels in TNBC.

As previous clinical and preclinical studies (Etienne *et al*, 1995; Salonga *et al*, 2000; Kobunai *et al*, 2007; Warnecke-Eberz *et al*, 2010) reported a beneficial effect of altered tumoural *DPYD* upon 5-FU treatment, we assessed patient outcome in the presence of *DPYD* CNVs. Remarkably, we observed prolonged progression-free intervals (TTP) associated with *DPYD* rearrangements in patients who had been treated with 5-FU- and/or anthracycline-based chemotherapy regimens. Furthermore, *DPYD* CNVs remained to be a strong parameter for predicting TTP independent of tumour stage. On the other hand, we could not confirm a potential benefit from DPD protein downregulation for adjuvant treatment. In contrast, our data revealed that absent/low (0–1+) DPD staining had no positive impact on TTP even in the subgroup treated with 5-FU-containing therapy only. This may be explained by our findings that, in TNBC, very low DPD expression was associated with more unfavourable clinical characteristics such as higher tumour stage (note that a large proportion of *DPYD* CNVs was associated with moderate DPD expression). Supporting these findings of an unfavourable clinical profile, analysis of publically available data sets of 125 hormone receptor-negative, basal-like breast cancers (supposed to be largely overlapping with TNBC), downloaded from GEO (Gyorffy *et al*, 2010), revealed that low *DPYD* mRNA expression was indeed related to shorter relapse-free survival (HR = 0.37 (0.2–0.7), log-rank $P = 0.0016$; Supplementary Figure S1).

On the basis of our results, the clinical impact of genomic *DPYD* rearrangements in TNBC appears to be mainly due to intrinsic tumour characteristics irrespective of the DPD expression status. Hence, *DPYD* CNVs may function as a surrogate marker predicting better clinical outcome upon radio- and chemotherapy. In fact, co-occurrence of *BRCA1* and *DPYD* rearrangements, as observed in our study, may reflect a background of decreased DNA repair capacity (Weigman *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

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.

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CONFLICT OF INTEREST

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

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