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Single-strand selective monofunctional uracil DNA glycosylase (SMUG1) deficiency is linked to aggressive breast cancer and predicts response to adjuvant therapy

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Abstract:	Uracil in DNA is an important cause of mutagenesis. SMUG1 is a uracil DNA glycosylase that removes uracil through base excision repair. SMUG1 also processes radiation induced oxidative base damage as well as 5-fluorouracil incorporated into DNA during chemotherapy. We investigated SMUG1 mRNA expression in 249 primary breast cancers. SMUG1 protein expression was investigated in 1165 breast tumours randomised into two cohorts [training set (n=583) and test set (n= 582)]. SMUG1 and chemotherapy response was also investigated in a series of 315 ER negative tumours (n=315). For mechanistic insights, SMUG1 was correlated to biomarkers of aggressive phenotype, DNA repair, cell cycle and apoptosis. Low SMUG1 mRNA expression was associated with adverse disease specific survival (p=0.008) and disease free survival (p=0.008). Low SMUG1 protein expression (25%) was associated with high histological grade (p<0.0001), high mitotic index (p<0.0001), pleomorphism (p<0.0001), glandular de-differentiation (p=0.0001), absence of hormonal receptors (ER-/PgR-/AR) (p<0.0001), presence of basal-like (p<0.0001) and triple negative phenotypes (p<0.0001). Low SMUG1 protein expression was associated with loss of BRCA1 (p<0.0001), ATM (p<0.0001) and XRCC1 (p<0.0001). Low p27 (p<0.0001), low p21 (p=0.023), mutant p53 (p=0.037), low MDM2 (p<0.0001), low MDM4 (p=0.004), low

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	Bcl-2 (p=0.001), low Bax (p=0.003) and high MIB1 (p<0.0001) were likely in low SMUG1 tumours. Low SMUG1 protein expression was associated with poor prognosis in univariate (p<0.001) and multivariate analysis (p<0.01). In ER+ cohort that received adjuvant endocrine therapy, low SMUG1 protein expression remains associated with poor survival (p<0.01). In ER- cohort that received adjuvant chemotherapy, low SMUG1 protein expression is associated with improved survival (p=0.043). Our study suggests that low SMUG1 expression may correlate to adverse clinicopathological features and predict response to adjuvant therapy in breast cancer.

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Single-strand selective monofunctional uracil DNA glycosylase (SMUG1) deficiency is linked to aggressive breast cancer and predicts response to adjuvant therapy

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ABSTRACT

Uracil in DNA is an important cause of mutagenesis. SMUG1 is a uracil DNA glycosylase that removes uracil through base excision repair. SMUG1 also processes radiation induced oxidative base damage as well as 5-fluorouracil incorporated into DNA during chemotherapy. We investigated SMUG1 mRNA expression in 249 primary breast cancers. SMUG1 protein expression was investigated in 1165 breast tumours randomised into two cohorts [training set (n=583) and test set (n= 582)]. SMUG1 and chemotherapy response was also investigated in a series of 315 ER negative tumours (n=315). For mechanistic insights, SMUG1 was correlated to biomarkers of aggressive phenotype, DNA repair, cell cycle and apoptosis. Low SMUG1 mRNA expression was associated with adverse disease specific survival (p=0.008) and disease free survival (p=0.008). Low SMUG1 protein expression (25%) was associated with high histological grade (p<0.0001), high mitotic index (p<0.0001), pleomorphism (p<0.0001), glandular de-differentiation (p=0.0001), absence of hormonal receptors (ER-/PgR-/AR) (p<0.0001), presence of basal-like (p<0.0001) and triple negative phenotypes (p<0.0001). Low SMUG1 protein expression was associated with loss of BRCA1 (p<0.0001), ATM (p<0.0001) and XRCC1 (p<0.0001). Low p27 (p<0.0001), low p21 (p=0.023), mutant p53 (p=0.037), low MDM2 (p<0.0001), low MDM4 (p=0.004), low Bcl-2 (p=0.001), low Bax (p=0.003) and high MIB1 (p<0.0001) were likely in low SMUG1 tumours. Low SMUG1 protein expression was associated with poor prognosis in univariate (p<0.001) and multivariate analysis (p<0.01). In ER+ cohort that received adjuvant endocrine therapy, low SMUG1 protein expression remains associated with poor survival (p<0.01). In ER- cohort that received adjuvant chemotherapy, low SMUG1 protein expression is associated with improved survival (p=0.043). Our study suggests that low SMUG1 expression may correlate to adverse clinicopathological features and predict response to adjuvant therapy in breast cancer.

Key words: DNA Base Excision Repair; SMUG1; breast cancer; prognostic factor; predictive factor.

INTRODUCTION

Uracil in DNA is an important cause of mutagenesis and may result either from incorporation of dUMP during replication leading to U:A mismatches, spontaneous generation or by enzymatic deamination of cytosine leading to U:G mismatches. Unrepaired U:G mismatches are 100% mutagenic leading to G:C to A:T transition mutations that are frequently seen in human tumours [1]. Uracil DNA repair is essential to protect against mutagenicity and this is accomplished by the DNA base excision repair (BER) machinery. Uracil BER is initiated by uracil DNA glycosylases. UNG2 and SMUG1 are important uracil DNA glycosylases that process uracil in DNA [1].

Emerging data suggests a role for SMUG1 in carcinogenesis. Smug1 was shown to be an important uracil-DNA glycosylase in Ung-deficient mice [2]. In Msh2(-/-) mice, loss of Smug1 as well as Ung increases cancer predisposition [3]. In addition, a 10-fold increase in spontaneous C:G to T:A transitions has been observed in cells deficient in Smug1 and Ung [4]. Smug1 and Ung deficient cells were also hypersensitive to ionizing radiation in that study [4]. A recent study has also demonstrated that in premenopausal women, SMUG1 rs2029166 genotype may increase breast cancer risk in those with low folate intake [5].

SMUG1 as well as UNG may also be essential for excising 5-fluorouracil (5-FU) incorporated into the DNA during chemotherapy. Although *Smug1* knockdown was shown to result in accumulation of 5-FU [6], the study however, did not take into consideration possible differences in growth rate or pool perturbation of nucleotide pool sizes. In a recent study, loss of UNG did not affect 5-FU sensitivity but loss of SMUG1 led to two fold increase in sensitivity to 24h treatment of 5-FU followed by recovery. In cell exposed to continuous 5-FU, however, no difference was observed in Smug1 depleted cells. Upon 5-FU treatment, SMUG1-depleted cells did show a prolonged S-phase

arrest and a transient increase in DNA double-strand breaks in that study [7]. Similarly, the role of Ung in 5-FU sensitivity has also been described in cell lines [8, 9]. SMUG1 is also a key enzyme for repairing 5-hydroxymethyluracil, 5-formyluracil, 5,6-dihydrouracil, alloxan and other lesions generated during oxidative base damage induced by ionising radiation and oxygen free radicals [4]. Removal of uracil in BER creates an abasic site (AP site) which is processed further by several enzymes including human AP endonuclease (APE1), poly (ADP-ribose) polymerase 1 (PARP1), DNA polymerase β and DNA ligase III-XRCC1 heterodimer which completes the repair process [10]. A recent study has also suggested a role for smug1 in RNA metabolism [11] implying additional functions for SMUG1.

Given the emerging role of SMUG1 in the maintenance of genomic integrity, we hypothesised that SMUG1 may be dysregulated in breast cancer. In the current study, we have investigated SMUG1 mRNA and protein expression in primary operable breast cancers and have demonstrated for the first time that SMUG1 deficiency may be linked to aggressive clinical phenotype and also predicts response to therapy.

MATERIALS AND METHODS

Study population

Uppsala cohort for SMUG1 mRNA expression: The Uppsala cohort originally composed of 315 women representing 65% of all breast cancers resected in Uppsala County, Sweden, from January 1, 1987, to December 31, 1989. Demographics are summarized in supplementary Table S1 of supporting information and also described elsewhere [12]. Tumour samples were microarray profiled on the Affymetrix U133A&B genechips. Microarray analysis was carried out at the Genome Institute of Singapore. All microarray data are accessible at National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Data can be accessed via series accession number (GSE4922). RNA preparation, microarray hybridization, and data processing were carried out essentially as described [13]. All data were normalized using the global mean method (MAS5), and probe set signal intensities were natural log transformed and scaled by adjusting the mean signal to a target value of log 500.

Nottingham cohort for SMUG1 protein expression: The study was performed in a consecutive series of patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series. A series of 1165 primary operable breast cancers were available. Patients were randomised into two equal cohorts by use of a double random number sort. Alternate cases were put into a training set (583 tumours) and a test set (583 tumours). All patients were treated uniformly in a single institution and have been investigated in a wide range of biomarker studies [14-16]. Supplemental Table S2 summarizes patient demographics. Both cohorts were well balanced with regards to clinicopathological features, treatment and survival data. Patients received standard surgery (mastectomy or wide local excision) with radiotherapy. Prior to 1989, patients did not receive systemic adjuvant treatment (AT). After 1989, AT was scheduled based on prognostic and predictive factor status, including Nottingham Prognostic Index (NPI), oestrogen receptor-α (ER-α) status, and menopausal status. Patients with

NPI scores of <3.4 (low risk) did not receive AT. Pre-menopausal patients with NPI scores of ≥3.4 (high risk) were given classical Cyclophosphamide, Methotrexate, and 5-Flourouracil (CMF) chemotherapy; patients with ER-α positive tumours were also offered hormone therapy (HT). Postmenopausal patients with NPI scores of ≥3.4 and ER-α positivity were offered HT, while ER-α negative patients received classical CMF chemotherapy. Median follow up was 111 months (range 1 to 233 months). Survival data, including overall survival, disease-free survival (DFS), and development of loco-regional and distant metastases (DM), was maintained on a prospective basis. DFS was defined as the number of months from diagnosis to the occurrence of local recurrence, local lymph node (LN) relapse or DM relapse. Breast cancer specific survival (BCSS) was defined as the number of months from diagnosis to the occurrence of BC related-death. Local recurrence-free survival (LRS) was defined the number of months from diagnosis to the occurrence of local recurrence. DM-free survival was defined as the number of months from diagnosis to the occurrence of DM relapse. Survival was censored if the patient was still alive at the time of analysis, lost to follow-up, or died from other causes.

We also evaluated a cohort of 315 ER-α negative invasive BCs. Demographic and treatment characteristics of this cohort are summarised in supplementary Table S3.

The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane et al [17], were followed throughout this study. This work was approved by Nottingham Research Ethics Committee.

Tissue Microarrays (TMAs) and immunohistochemistry (IHC)

Tumours were arrayed in tissue microarrays (TMAs) constructed with 2 replicate 0.6mm cores from the centre and periphery of the tumours. The TMAs were immunohistochemically profiled for SMUG1 and other biological antibodies [ER, PR, AR, Her-2, CK5/6, CK14, EGFR, ATM, BRCA1, XRCC1, P27, P21, MIB1, Bax, BCL-2, p 53, MDM2 and MDM4 as previously described [18-21] (primary antibodies, clone, source, optimal dilution and scoring system used for each

immunohistochemical marker is summarized in supplementary Table S4)]. Immunohistochemical staining for SMUG1 was performed using the Bond Max automated staining machine and Leica Bond Refine Detection kit (DS9800) according to manufacturer instructions (Leica Microsystems). TMAs sections were incubated for 15 minutes at room temperature with 1/200 goat anti-SMUG1 monoclonal antibody (Acris Antibody GmbH). Pre-treatment of TMA section was performed with citrate buffer (pH 6.0) for 20 minutes. HER2 expression was assessed according to the new ASCO/CAP guidelines using IHC and fluorescence in situ hybridisation (FISH) [22].

Evaluation of immune staining: We utilised H-score method as well as quick score method for SMUG1 nuclear staining analysis in tumours. For H-score assessment, whole field inspection of the core was performed and intensities of nuclear staining were grouped as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. The percentage of staining in each category was estimated (0-100%). H-score (range 0-300) was calculated by multiplying intensity of staining by percentage staining as previously described [18-21]. Low SMUG1 (SMUG1-) expression was defined by mean H-score ≤35.

For quick-score, the intensity and proportion of cells staining for SMUG1 was analysed. Proportion of staining was scored as follows: 0 (negative), 1 (≤1%), 2 (1-10%), 3 (11-33%), 4 (34-66%) and 5 (>66-100%). Intensity of staining was scored as follows: 1 (weak), 2 (moderate) and 3 (strong). The two scores were added to give a quick-score in the range 0-8 (by definition, there is no quick-score of 1) [19]. Low SMUG1 (SMUG1-) expression was defined by Quick score < 4 which was equivalent to H-score ≤35. Not all cores within the TMA were suitable for IHC analysis due to missing cores or absence of tumour cells.

Cancer cell lines and culture: In addition to the breast cancer cell lines **(**MCF-7, MDA-MB-231, MDA-MB-436), we also investigated colon cancer cell lines (Colo-205, C-170), gastric cancer cell lines (AGS, ST-16) and pancreatic cancer cell lines (Panc1, ASPC-1) for SMUG1 protein expression. The cell lines were purchased from ATCC and grown in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin.

Western blot analysis: This assay was performed as described previously [23]. Primary antibodies used was an anti-SMUG1 goat polyclonal antibody (catalogue no: AP08884PU-N, Acris Antibody GmbH, Germany). The secondary antibody was a HRP conjugated secondary anti-goat (Dako, Glostrup, Denmark) antibody. As well as cell extracts from the cell lines, recombinant GST-tagged SMUG1 protein (Novus biological, USA) was simultaneously used as a positive control and to investigate the specificity of anti-SMUG1 antibody .

Statistical analysis: Data analysis was performed using SPSS (SPSS, version 17 Chicago, IL). Where appropriate, Pearson's Chi-square, Fisher's exact, Student's t and ANOVA one-way tests were used. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazard model. The proportional hazards assumption was tested using standard log-log plots. Hazard ratio (HR) and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI and a p value < 0.05 considered significant. For multiple comparisons, p values were adjusted according to Holm-Bonferroni correction method [24].

RESULTS

Low SMUG1 transcript levels correlate to poor survival

We first evaluated SMUG1 mRNA expression in 249 breast cancers comprising the Uppsala cohort. 50.6% of tumours had high SMUG1 mRNA levels and 49.4% of tumours had low SMUG1 mRNA expression levels. Low SMUG1 mRNA expression in tumours was associated with adverse disease specific survival (p=0.008) (Figure 1A) and disease free survival (p=0.008) (Figure 1B) in patients.

We then proceeded to investigate SMUG1 protein expression in breast cancer.

Low SMUG1 protein expression is linked to aggressive phenotype

We evaluated specificity of SMUG1 antibody by western blot analysis. In addition to the breast cancer cell lines **(**MCF-7, MDA-MB-231, MDA-MB-436), we also investigated colon cancer cell lines (Colo-205, C-170), gastric cancer cell lines (AGS, ST-16) and pancreatic cancer cell lines (Panc1, ASPC-1) for SMUG1 protein expression. Recombinant SMUG1 protein was used as a positive control. Figure 2A demonstrates robust SMUG1 protein expression across a panel of cancer cell lines. The anti-SMUG1 antibody that specifically binds recombinant SMUG1 protein was used as a positive control and provides evidence for the specificity of the antibody. In addition, Figure 2B demonstrates more than two-fold reduction in SMUG1 expression in MDA-MB-436 cells compared to MCF-7 cells providing evidence of differential expression of SMUG1 across breast cancer cell lines. We then proceeded to conduct immunohistochemical (IHC) evaluation of SMUG1 protein expression in human breast cancers. We first investigated in a training set and then validated in a test set of breast cancer cohorts.

a) Training set (n=583):145/583 (25%) of the tumours were low for SMUG1 expression, and 439/583 (75%) tumours were high for SMUG1 expression (Figure 2E and 2F). Low SMUG1 expression was highly significantly associated with adverse pathological features (Table 1)

including high histological grade (p<0.0001), high mitotic index (p<0.0001), pleomorphism (p<0.0001) and glandular de-differentiation (p=0.0001). In addition, low SMUG1 expression was significantly linked to aggressive phenotypic features such as absence of hormonal receptors (ER-/PgR-/AR) (p<0.0001), EGFR over expression (p=0.024), presence of basal like phenotype (p<0.0001) and triple negative phenotype (p<0.0001) (Table 1).

Low SMUG1 expression was significantly associated with loss of expression of BRCA1 (p<0.0001), ATM (p<0.0001) and XRCC1 (p<0.0001). Loss of p27 and p21 expression was more common in low SMUG1 tumours (p<0.0001 and p=0.023 respectively). High MIB1 was significantly associated with low SMUG1 expression (p<0.0001). Low SMUG1 expression was also significantly associated with low expression of p53 downstream genes that regulate cell cycle progression and apoptosis such as MDM2 (p=0.016), MDM4 (p=0.0003), Bcl2 (p<0.0001) and Bax (p=0.008).

In the full cohort, low SMUG1 expression is associated with adverse clinical outcome at 10 years, with a significant increase in the risk of death (p<0.0001) (Figure 2G) and recurrence (p=0.005) (Figure 2H) compared with high SMUG1 tumours. Low SMUG1 ER + breast cancers receiving adjuvant endocrine therapy had poor survival (p=0.023) and a trend to increased recurrence (p=0.099) compared to patients with high SMUG1 tumours (Figures 3A and 3B).

In multivariate Cox regression analysis including other validated prognostic factors (such as lymph node stage, histological grade and tumour size), low SMUG1 was an independent predictor for breast cancer specific survival (p= 0.018) as well as disease free survival (p=0.031) (Table 3).

b) Test set (n= 582): 138/582 (24%) of the tumours were low for SMUG1 expression, and 444/582 (76%) tumours were high for SMUG1 expression. Low SMUG1 expression was highly significantly associated with adverse pathological features (Table 1) including larger tumours (p=0.009), high histological grade (p<0.0001), high mitotic index (p<0.0001), pleomorphism (p<0.0001) and glandular de-differentiation (p=0.0042). In addition, low SMUG1 expression was

also significantly linked to aggressive features such as absence of hormonal receptors (ER-/PgR-/AR) ($p<0.0001$), EGFR over expression ($p=0.002$), HER-2 overexpression ($p=0.038$), presence of basal like phenotype ($p<0.0001$) and triple negative phenotype ($p<0.0001$) (Table 1).

Low SMUG1 expression was significantly associated with loss of expression of BRCA1 ($p=0.01$), ATM ($p=0.002$) and XRCC1 ($p<0.0001$). Loss of p27 was more common in low SMUG1 tumours ($p<0.0001$). High MIB1 was significantly associated with low SMUG1 tumours ($p<0.0001$). Low SMUG1 expression was also significantly associated with mutant p53 ($p=0.037$) as well as low expression of p53 downstream genes that regulate cell cycle progression and apoptosis such as MDM2 ($p<0.0001$), MDM4 ($p=0.004$), Bcl2 ($p=0.001$) and Bax ($p=0.003$).

In the full cohort, low SMUG1 expression is associated with adverse clinical outcomes at 10 years with a significant increase in the risk of death ($p<0.0001$) (Figure 2I) and recurrence ($p=0.006$) (Figure 2J) compared with SMUG1 high tumours. Low SMUG1 ER+ breast cancers receiving adjuvant endocrine therapy had poor survival ($p=0.003$) and increased recurrence ($p=0.016$) compared to patients with SMUG1 high tumours (Figures 3C and 3D).

In multivariate Cox regression analysis including other validated prognostic factors (such as lymph node stage, histological grade and tumour size), low SMUG1 expression was an independent predictor for breast cancer specific survival ($p= 0.027$) as well as disease free survival ($p=0.034$) (Table 3).

Clinicopathological significance in ER negative breast cancers (n= 315)

77/315 (24%) of the tumours were low for SMUG1 expression, and 238/315 (76%) tumours were high for SMUG1 expression. Low SMUG1 expression was significantly associated with high vascular invasion ($p=0.01$) and triple negative phenotype ($p=0.003$) (Table 4). Her-2 over expression ($p=0.001$) and lymph node positive disease ($p=0.008$) was more likely in SMUG1 high tumours. Low SMUG1 expression was more likely to be associated with XRCC1 loss in this cohort.

In ER- patients who received chemotherapy, high SMUG1 expression was associated with 2-fold increase in risk of death (p=0.043) and recurrence (p=0.043) indicating resistance to chemotherapy (Figures 3E and 3F).

DISCUSSION

Impaired DNA repair may increase mutation rate, enhance chromosomal instability and promote selection of more malignant clones with aggressive behaviour [25, 26]. SMUG1 uracil DNA glycosylase may be an anti-mutator protein involved in BER [2]. Besides a role in genomic integrity, a recent study has also suggested a critical role for smug1 in RNA metabolism [11]. This is the first study to evaluate SMUG1 expression in breast cancer. We provide the first evidence that low SMUG1 expression in breast tumours is associated with an aggressive phenotype such as high histological grade, pleomorphism, glandular de-differentiation, absence of hormonal receptors and presence of basal like and triple negative phenotypes. Low SMUG1 expression is also associated with loss of expression of BRCA1, ATM and XRCC1, implying genomic instability in SMUG1 low tumours. Moreover, association with abnormal expression of p53, p27, MDM2, MDM4, Bcl-2 and Bax provides additional evidence for higher level of genomic instability in SMUG1 low tumours. Low SMUG1 expression was associated with poor survival in univariate as well as multivariate analysis in both the training and test set that predominantly consisted of ER + tumours (80%). In ER+ tumours that received endocrine therapy, in particular, low SMUG1 expression is also associated with poor survival indicating that SMUG1 status may predict endocrine response, although the mechanism for resistance is unknown. On the other hand, in ER- tumours that received chemotherapy, low SMUG1 expression is associated with better survival indicating sensitivity to chemotherapy. The clinical data presented in ER- tumours is consistent with preclinical studies where SMUG1 depletion has been shown to result in sensitivity to 5-FU chemotherapy [6, 27].

In a recent study in 112 gastric cancers, we found that SMUG1 high tumours were associated with adverse clinical outcome such as poor disease free survival (p=0.02) and disease specific survival (p=0.05) [28]. The data in gastric cancer is in contrast to that presented in breast tumours in the current study. We speculate that low SMUG1 expression in breast cancer may increase genomic

instability and promote a cancerous phenotype. On the other hand, in gastric cancer, where inflammation is driver for carcinogenesis, up-regulation of BER may be required to repair oxidative base damage that is commonly seen in an inflammatory environment. SMUG1 up-regulation in this context could promote cancer survival and resistance to therapy. Taken together, the data suggests that SMUG1 may have complex roles in carcinogenesis and larger studies in multiple tumour types are required to clarify further the role of SMUG1 in cancer. A limitation to our study is that it is retrospective. Although we have demonstrated that low SMUG1 is associated with an aggressive phenotype, we have not directly shown that SMUG1 loss results in a mutator phenotype in breast cancer. Aggressive tumours are highly proliferative and it is possible that SMUG1 down-regulation in highly proliferative tissue may have no causal relation to the aggressive phenotype. Future mechanistic preclinical studies could clarify whether SMUG1 loss confers a mutator phenotype in breast cancer.

The link between DNA repair, ER, p53 and its downstream targets MDM2 and p21 in breast cancer are beginning to emerge [29]. Although the regulation of uracil DNA glycosylases is largely unknown, recent studies suggest a potential role for p53. For example, thymine DNA glycosylase (TDG) that belongs to the superfamily of uracil DNA glycosylases, has been shown to be transcriptionally regulated by p53 [30]. Moreover, PPM1D, a p53-induced oncogenic phosphatase has been shown to interact with uracil DNA glycosylase (UNG2) and suppress BER [31]. Interestingly, p73, a member of p53 family, may be directly involved in transcriptional regulation of SMUG1 [32]. Whether p53 is also involved in SMUG1 regulation remains unknown. In addition, NFI/CTF transcription factor has also been shown to be involved in SMUG1 regulation [33] implying that SMUG1 regulation may be complex in cells.

We recently investigated XRCC1, another key BER protein in breast cancer [23]. As seen in SMUG1, loss of XRCC1 (16%) was also associated with high grade ($p<0.0001$), loss of hormone receptors ($p<0.0001$), triple negative ($p<0.0001$) and basal like phenotypes ($p=0.001$). Loss of XRCC1 was associated with a 2-fold increase in risk of death ($p<0.0001$) and independently with

poor outcome ($p<0.0001$). We also demonstrated a novel synthetic lethality application using DNA double strand break repair inhibitors in XRCC1 deficient breast cancer cells [23]. Taken together, our data suggests that BER deficiency in breast tumours contribute to aggressive clinical behaviour and could be targeted for personalized treatment strategy in patients.

Conflict of interest: All authors declared no conflict of interest that could be perceived as prejudicing the impartiality of the research reported and no financial or other potential conflict of interest.

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REFERENCES

1. T. Visnes, B. Doseth, H. S. Pettersen, L. Hagen, M. M. Sousa, M. Akbari, et al. (2009) Uracil in DNA and its processing by different DNA glycosylases. Philos Trans R Soc Lond B Biol Sci 364 (1517):563-8. doi: 10.1098/rstb.2008.0186

2. H. Nilsen, K. A. Haushalter, P. Robins, D. E. Barnes, G. L. Verdine and T. Lindahl (2001) Excision of deaminated cytosine from the vertebrate genome: role of the SMUG1 uracil-DNA glycosylase. Embo J 20 (15):4278-86.

3. K. Kemmerich, F. A. Dingler, C. Rada and M. S. Neuberger Germline ablation of SMUG1 DNA glycosylase causes loss of 5-hydroxymethyluracil- and UNG-backup uracil-excision activities and increases cancer predisposition of Ung-/-Msh2-/- mice. Nucleic Acids Res 40 (13):6016-25. doi: 10.1093/nar/gks259

4. Q. An, P. Robins, T. Lindahl and D. E. Barnes (2005) C --> T mutagenesis and gamma-radiation sensitivity due to deficiency in the Smug1 and Ung DNA glycosylases. Embo J 24 (12):2205-13.

5. C. Marian, M. Tao, J. B. Mason, D. S. Goerlitz, J. Nie, A. Chanson, et al. Single nucleotide polymorphisms in uracil-processing genes, intake of one-carbon nutrients and breast cancer risk. Eur J Clin Nutr 65 (6):683-9. doi: 10.1038/ejcn.2011.29

6. Q. An, P. Robins, T. Lindahl and D. E. Barnes (2007) 5-Fluorouracil incorporated into DNA is excised by the Smug1 DNA glycosylase to reduce drug cytotoxicity. Cancer Res 67 (3):940-5

7. P. Nagaria, D. Svilar, A. R. Brown, X. H. Wang, R. W. Sobol and M. D. Wyatt SMUG1 but not UNG DNA glycosylase contributes to the cellular response to recovery from 5-fluorouracil induced replication stress. Mutat Res 743-744:26-32. doi: 10.1016/j.mrfmmm.2012.12.001

8. H. S. Pettersen, T. Visnes, C. B. Vagbo, E. K. Svaasand, B. Doseth, G. Slupphaug, et al. UNG-initiated base excision repair is the major repair route for 5-fluorouracil in DNA, but 5-fluorouracil cytotoxicity depends mainly on RNA incorporation. Nucleic Acids Res 39 (19):8430-44. doi: 10.1093/nar/gkr563

9. B. C. Grogan, J. B. Parker, A. F. Guminski and J. T. Stivers Effect of the thymidylate synthase inhibitors on dUTP and TTP pool levels and the activities of DNA repair glycosylases on uracil and 5-fluorouracil in DNA. Biochemistry 50 (5):618-27. doi: 10.1021/bi102046h

10. R. Abbotts and S. Madhusudan Human AP endonuclease 1 (APE1): from mechanistic insights to druggable target in cancer. Cancer Treat Rev 36 (5):425-35. doi: 10.1016/j.ctrv.2009.12.006

11. L. Jobert, H. K. Skjeldam, B. Dalhus, A. Galashevskaya, C. B. Vagbo, M. Bjoras, et al. The human base excision repair enzyme SMUG1 directly interacts with DKC1 and contributes to RNA quality control. Mol Cell 49 (2):339-45. doi: 10.1016/j.molcel.2012.11.010

12. J. Bergh, T. Norberg, S. Sjogren, A. Lindgren and L. Holmberg (1995) Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. Nat Med 1 (10):1029-34

13. Y. Pawitan, J. Bjohle, L. Amler, A. L. Borg, S. Egyhazi, P. Hall, et al. (2005) Gene expression profiling spares early breast cancer patients from adjuvant therapy: derived and validated in two population-based cohorts. Breast Cancer Res 7 (6):R953-64

14. C. W. Elston and I. O. Ellis (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19 (5):403-10
15. I. O. Ellis, M. Galea, N. Broughton, A. Locker, R. W. Blamey and C. W. Elston (1992) Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up. Histopathology 20 (6):479-89
16. M. H. Galea, R. W. Blamey, C. E. Elston and I. O. Ellis (1992) The Nottingham Prognostic Index in primary breast cancer. Breast Cancer Res Treat 22 (3):207-19
17. L. M. McShane, D. G. Altman, W. Sauerbrei, S. E. Taube, M. Gion and G. M. Clark (2005) Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 97 (16):1180-4
18. T. M. Abdel-Fatah, D. G. Powe, G. Ball, M. A. Lopez-Garcia, H. O. Habashy, A. R. Green, et al. Proposal for a modified grading system based on mitotic index and Bcl2 provides objective determination of clinical outcome for patients with breast cancer. J Pathol 222 (4):388-99. doi: 10.1002/path.2775
19. T. M. Abdel-Fatah, D. G. Powe, J. Agboola, M. Adamowicz-Brice, R. W. Blamey, M. A. Lopez-Garcia, et al. The biological, clinical and prognostic implications of p53 transcriptional pathways in breast cancers. J Pathol 220 (4):419-34. doi: 10.1002/path.2663
20. G. M. Callagy, P. D. Pharoah, S. E. Pinder, F. D. Hsu, T. O. Nielsen, J. Ragaz, et al. (2006) Bcl-2 is a prognostic marker in breast cancer independently of the Nottingham Prognostic Index. Clin Cancer Res 12 (8):2468-75
21. D. S. Tan, C. Marchio, R. L. Jones, K. Savage, I. E. Smith, M. Dowsett, et al. (2008) Triple negative breast cancer: molecular profiling and prognostic impact in adjuvant anthracycline-treated patients. Breast Cancer Res Treat 111 (1):27-44
22. A. C. Wolff, M. E. Hammond, J. N. Schwartz, K. L. Hagerty, D. C. Allred, R. J. Cote, et al. (2007) American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 25 (1):118-45
23. R. Sultana, T. Abdel-Fatah, R. Abbotts, C. Hawkes, N. Albarakati, C. H. Seedhouse, et al. (2013) Targeting XRCC1 deficiency in breast cancer for personalized therapy. Cancer Res 73(5):1621-34. doi: 10.1158/0008-5472.CAN-12-2929
24. S. Holm (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6:65-70.
25. R. A. Beckman and L. A. Loeb (2006) Efficiency of carcinogenesis with and without a mutator mutation. Proc Natl Acad Sci U S A 103 (38):14140-5
26. L. A. Loeb, J. H. Bielas and R. A. Beckman (2008) Cancers exhibit a mutator phenotype: clinical implications. Cancer Res 68 (10):3551-7; discussion 3557. doi: 10.1158/0008-5472.CAN-07-5835
27. P. Nagaria, D. Svilar, A. R. Brown, X. H. Wang, R. W. Sobol and M. D. Wyatt (2013) SMUG1 but not UNG DNA glycosylase contributes to the cellular response to recovery from 5-fluorouracil induced replication stress. Mutat Res 743:26-32. doi: 10.1016/j.mrfmmm.2012.12.001.
28. T. Abdel-Fatah, A. Arora, I. Gorguc, R. Abbotts, S. Beebeejaun, S. Storr, et al. (2013) Are DNA Repair Factors Promising Biomarkers for Personalized Therapy in Gastric Cancer? Antioxid Redox Signal 18(18):2392-8. doi: 10.1089/ars.2012.4873
29. C. E. Berger, Y. Qian, G. Liu, H. Chen and X. Chen (2012) p53, a target of estrogen receptor (ER) alpha, modulates DNA damage-induced growth suppression in ER-positive breast cancer cells. J Biol Chem 287 (36):30117-27. doi: 10.1074/jbc.M112.367326

30. N. M. da Costa, A. Hautefeuille, M. P. Cros, M. E. Melendez, T. Waters, P. Swann, et al. (2012) Transcriptional regulation of thymine DNA glycosylase (TDG) by the tumor suppressor protein p53. Cell Cycle 11 (24):4570-8. doi: 10.4161/cc.22843
31. X. Lu, D. Bocangel, B. Nannenga, H. Yamaguchi, E. Appella and L. A. Donehower (2004) The p53-induced oncogenic phosphatase PPM1D interacts with uracil DNA glycosylase and suppresses base excision repair. Mol Cell 15 (4):621-34
32. E. Zaika, J. Wei, D. Yin, C. Andl, U. Moll, W. El-Rifai, et al. (2011) p73 protein regulates DNA damage repair. Faseb J 25 (12):4406-14. doi: 10.1096/fj.11-192815
33. I. Elateri, S. Muller-Weeks and S. Caradonna (2003) The transcription factor, NFI/CTF plays a positive regulatory role in expression of the hSMUG1 gene. DNA Repair (Amst) 2 (12):1371-85

Table 1. Association between SMUG expression and clinicopathologic variables in the training set

Variable	SMUG1 expression		
	Low	High	Adjusted p value
A) Pathological Parameters			
Tumour Size			**0.043**
T1 a + b (≤1.0)	9 (6.3)	53 (12.2)	
T1 c (>1.0 -2.0)	67 (46.9)	219 (50.5)	
T2 (>2.0-5)	63 (44.1)	158 (36.4)	
T3 (>5)	4 (2.8)	4 (0.9)	
Lymph node stage			0.223
Negative	81 (56.6)	266 (61.1)	
Positive (1-3 nodes)	52 (36.4)	127 (29.2)	
Positive (>3 nodes)	10 (7)	42 (9.7)	
Grade**			**<1 x 10^{-6}**
G1	15 (10.5)	78 (18)	
G2	23 (16.1)	180 (41.5)	
G3	105 (73.4)	176 (40.6)	
Tumour Types			**0.003**
IDC-NST	88 (72.7)	214 (54.2)	
Tubular	16 (13.2)	90 (22.8)	
ILC	5 (4.1)	10 (2.5)	
Medullary	5 (4.1)	10 (2.5)	
Others	7 (5.8)	39 (9.9)	
Mitotic Index			**1 x 10^{-6}**
M1 (low; mitoses < 10)	20 (14.4)	180 (41.6)	
M2 (medium; mitoses 10-18)	23 (16.5)	97 (22.4)	
M3 (high; mitosis >18)	96 (69.1)	156 (36)	
Pleomorphism			
1 (small-regular uniform)	1 (0.7)	15 (3.5)	**1 x 10^{-6}**
2 (Moderate variation)	33 (23.7)	199 (46)	
3 (Marked variation)	105 (75.5)	219 (50.6)	
Tubule formation			**1.8 x 10^{-3}**
1 (>75% of definite tubule)	2 (1.4)	31 (7.2)	
2 (10%-75% definite tubule)	38 (27.3)	159 (36.7)	
3 (<10% definite tubule)	99 (71.2)	243 (56.1)	
B) Aggressive phenotype			
EGFR expression			**0.024**
Low	78 (72.9)	289 (82.8)	
High	29 (27.1)	60 (17.2)	
Her2 overexpression			0.502
No	121 (87.7)	384 (89.7)	
Yes	17 (12.3)	44 (10.3)	
Triple negative			**1 x 10^{-6}**
No	89 (64)	372	
Yes	50 (36)	53 (12.5)	
Basal phenotype			**7 x 10^{-5}**
No	95 (76)	378 (89.8)	
Yes	30 (24)	43 (10.2)	
C) Hormone receptors			
ER			**< 1 x 10^{-6}**
Negative	66 (46.5)	84 (19.8)	
Positive	76 (53.5)	341 (80.2)	
PgR			**6.9 x 10^{-5}**
Negative	76 (56.7)	150 (37.1)	
Positive	58 (43.3)	254 (62.9)	

AR			**9.5 x 10 $^{-5}$**
Negative	55 (50)	104 (29.7)	
Positive	55 (50)	246 (70.3)	
D)DNA Repair			
ATM expression			**2.6 x 10 $^{-4}$**
Negative	67 (69.8)	128 (48.1)	
Positive	29 (30.2)	138 (51.9)	
BRCA 1 expression			**1 x 10 $^{-6}$**
Negative	44 (45.8)	41 (13.1)	
Positive	52 (54.2)	271 (86.9)	
XRCC1 expression			**< 1 x 10 $^{-6}$**
Low	46 (36.3)	30 (8.6)	
High	71 (60.7)	319 (91.4)	
E) Cell cycle/apoptosis regulators			
p27 expression			**8 x 10 $^{-4}$**
Low	76 (80.9)	180 (59.6)	
High	18 (19.1)	121 (40.1)	
p21 expression			**0.023**
Negative	68 (63)	174 (50.4)	
Positive	40 (37)	171 (49.6)	
MIB1			**<1 x 10 $^{-6}$**
Low	21 (17.9)	155 (42.8)	
High	96 (82.1)	207 (57.2)	
Bcl-2 expression			**< 1 x 10 $^{-6}$**
No	63 (51.2)	98 (24.6)	
Yes	60 (48.8)	301 (75.4)	
Bax expression			**0.008**
Low	76 (82.6)	188 (68.1)	
High	16 (17.4)	88 (31.9)	
P53 expression			0.123
Low	86 (76.1)	295 (82.6)	
High	27 (23.9)	62 (17.4)	
MDM2 expression			**0.016**
Low	87 (85.3)	241 (73.7)	
High	15 (14.7)	86 (26.3)	
MDM4 expression			**3.2 x 10 $^{-3}$**
Low	88 (93.6)	240 (80.8)	
High	6 (6.4)	57 (19.2)	

Table 2. Association between SMUG1 expression and clinicopathologic variables in the test set

Variable	SMUG1 expression Low N(%)	High N(%)	Adjusted p value
A) Pathological Parameters			
Tumour Size			**0.009**
T1 a + b (≤1.0)	13 (9.4)	52 (11.8)	
T1 c (>1.0 -2.0)	65 (47.1)	224 (50.9)	
T2 (>2.0-5)	51 (37)	158 (35.9)	
T3 (>5)	9 (6.5)	6 (1.4)	
Lymph node stage			0.223
Negative	81 (56.6)	266 (61.1)	
Positive (1-3 nodes)	52 (36.4)	127 (29.2)	
Positive (>3 nodes)	15 (10.9)	36 (8.2)	
Grade**			**< 1 X 10 $^{-6}$**
G1	15 (10.5)	78 (18)	
G2	23 (16.1)	180 (41.5)	
G3	105 (73.4)	176 (40.6)	
Tumour Types			**0.045**
IDC-NST	82 (69.5)	220 (55.1)	
Tubular	17 (14.4)	91 (22.8)	
ILC	9 (7.6)	50 (12.5)	
Medullary	4 (3.4)	8 (2)	
Others	6 (5.1)	30 (7.5)	
Mitotic Index			**2 X 10 $^{-6}$**
M1 (low; mitoses < 10)	26 (19)	180 (41)	
M2 (medium; mitoses 10-18)	24 (17.5)	80 (41)	
M3 (high; mitosis >18)	87 (63.5)	179 (40.8)	
Pleomorphism			**1 X 10 $^{-6}$**
1 (small-regular uniform)	1 (0.7)	11 (2.5)	
2 (Moderate variation)	28 (0.4)	193 (44.1)	
3 (Marked variation)	108 (78.8)	234 (53.4)	
Tubule formation			**0.042**
1 (>75% of definite tubule)	3 (2.2)	30 (6.8)	
2 (10%-75% definite tubule)	39 (28.5)	147 (33.5)	
3 (<10% definite tubule)	95 (69.3)	262 (59.7)	
B) Aggressive phenotype			
EGFR expression			**0.002**
Low	84 (75)	313 (87.2)	
High	28 (25)	46 (12.8)	
Her2 overexpression			**0.038**
No	116 (84.1)	396 (90.4)	
Yes	22 (15.9)	42 (9.6)	
Triple negative			**4.9 x 10 $^{-6}$**
No	96 (07.1)	365 (85.5)	
Yes	41 (29.9)	62 (14.5)	
Basal phenotype			**3.2 X 10 $^{-4}$**
No	109 (81.3)	391 (92.2)	
Yes	25 (18.7)	33 (7.8)	
C) Hormonal receptors			
ER			**3 x 10 $^{-6}$**
Negative	58 (42)	94 (21.7)	
Positive	80 (58)	339 (78.3)	
PgR			**5 x 10 $^{-6}$**
Negative	77 (57.5)	145 (35.2)	
Positive	57 (42.5)	267 (64.8)	

AR			**3.2 X 10 $^{-4}$**
Negative	57 (52.3)	121 (33.2)	
Positive	52 (47.7)	243 (66.8)	
D) DNA Repair			
ATM expression			**0.002**
Negative	55 (66.3)	117 (46.4)	
Positive	28 (33.7)	135 (53.6)	
BRCA 1 expression			**0.01**
Negative	29 (27.9)	54 (16.4)	
Positive	75 (72.1)	275 (83.6)	
XRCC1 expression			**< 1 x 10 $^{-6}$**
Low	34 (31.5)	39 (10.9)	
High	74 (68.5)	320 (89.1)	
E) Cell cycle/apoptosis regulators			
p27 expression			**4.5 x 10 $^{-5}$**
Low	78 (80.4)	154 (54.8)	
High	19 (19.6)	126 (44.8)	
p21 expression			0.212
Negative	64 (64)	195 (57)	
Positive	36 (36)	147 (43)	
MIB1			**7.8 x 10 ^{5}**
Low	22 (19)	145 (38.9)	
High	94 (81)	228 (61.1)	
Bcl-2 expression			**0.001**
Low	63 (50)	142 (34.1)	
High	63 (50)	275 (65.9)	
Bax expression			**0.003**
Low	77 (81.9)	188 (65.5)	
High	17 (18.1)	99 (34.5)	
P53 expression			**0.037**
Low	80 (72.1)	315 (81.2)	
High	31 (27.9)	73 (18.8)	
MDM2 expression			**5 X 10 $^{-6}$**
Low	97 (94.2)	233 (72.8)	
High	6 (5.8)	87 (27.2)	
MDM4 expression			**0.004**
Low	91 (93.8)	239 (81.8)	
High	6 (6.2)	53 (18.2)	

Table 3. Multivariate Cox regression analysis of both training and test sets

Variables	BCSS at 10 years						DFS at 10 years					
	Training Set			Test Set			Training Set			Test Set		
	HR	CI 95%	p	HR	CI 5%	p	HR	CI 95%	p	HR	CI 95%	p
Low SMUG1	1.5	1.08-2.20	**0.018***	1.5	1.05-2.06	**0.027***	1.4	1.03-1.91	**0.031***	1.3	1.03-1.83	**0.034***
Lymph node stage			**0.00005***			**$5.7x10^{-10*}$**			**0.002***			**$1.2x10^{-10*}$**
Negative	1			1			1			1		
Positive (1-3 nodes)	1.8	1.25-2.54		1.2	0.84-1.78		1.4	1.02-1.88		1.2	0.91-1.65	
Positive (>3 nodes)	2.8	1.71-4.64		4.1	2.65-6.31		2.2	1.37-3.43		3.6	2.48-5.27	
Grade**			**0.001***			**$2.0x10^{-9*}$**			0.085			**0.018***
G1	1				1		1	1		1	1	
G2	1.5	0.74-2.87		2.5	1.04-6.10		0.8	0.53-1.30		1.4	0.90-2.16	
G3	2.5	1.32-4.73		7.0	3.04-15.9		1.2	0.78-1.83		1.8	1.17-2.69	
Size (continuous)	1.3	1.12-1.49	**0.0005***	1.1	1.01-1.24	**0.038***	1.2	1.05-1.37	**0.006***	1.1	1.04-1.25	**007***

Table 4: Association between SMUG1 expression and clinicopathological variables in ER negative cohort

Variable	SMUG1 expression		Adjusted p value
	Low (n= 77)	High (n= 238)	
			0.424
T1 a + b (≤1.0)	5 (6.8)	27 (11.6)	
T1 c (>1.0 -2.0)	30 (41.1)	101(43.5)	
T2 (>2.0-5)	35 (47.9)	90 (38.8)	
T3 (>5)	3 (4.1)	14 (6)	
Grade**			0.257
G1	0	0	
G2	5 (6.5)	26 (10.9)	
G3	72 (93.5)	212 (89.1)	
Lymph node metastasis			**0.008***
No	60 (77.9)	146 (61.3)	
Yes	17 (22.1)	92 (38.7)	
Mitotic Index			**0.028****
M1 (low; mitoses < 10)	3 (3.9)	17 (7.2)	
M2 (medium; mitoses 10-18)	7 (9.2)	50 (21.1)	
M3 (high; mitosis >18)	66 (86.8)	170 (71.7)	
Lympho-vascular invasion			**0.01***
No	13 (17.1)	77 (32.5)	
Yes	63 (82.9)	160 (67.5)	
Pleomorphism			0.324
1 (small-regular uniform)	0	3 (1.3)	
2 (Moderate variation)	76 (100)	234 (98.7)	
3 (Marked variation)	33 (45.8)	106 (49.3)	
Tubule formation			0.672
1 (>75% of definite tubule)	0	1 (0.4)	
2 (10%-75% definite tubule)	8 (10.5)	32 (13.5)	
3 (<10% definite tubule)	68 (89.5)	204 (86.1)	
Her2 overexpression			**0.001***
No	72 (93.5)	177 (75.6)	
Yes	5 (6.5)	57 (24.4)	
Triple negative			**0.003***
No	7 (9.1)	58 (24.8)	
Yes	70 (90.9)	176 (75.2)	
ATM expression			0.712
Negative	40 (64.5)	117 (61.9)	
Positive	22 (35.5)	72 (38.1)	
BRCA 1 expression			
Negative	59 (88.1)	186 (92.1)	0.317
Positive	8 (11.9)	16 (7.9)	
XRCC1 expression			
Low	19 (26.4)	29 (12.6)	**0.005***
High	53 (73.6)	202 (87.4)	

FIGURE LEGENDS

Figure 1: SMUG1 mRNA expression in breast cancer. **A.** Kaplan Meier curves showing breast cancer specific survival (BCSS). **B**. Disease free survival (DFS) in the training set (**C**)

Figure 2: SMUG1 protein expression in breast cancer**. A.** Western blot of recombinant SMUG1 protein and cell extract from a panel of cancer cell lines (see text for details). **B.** Western blot demonstrating more than two-fold reduction in SMUG1 expression in MDA-MB-436 cells compared to MCF-7 cells. **C.** Microphotograph of SMUG1 high and low breast cancer tissue. Kaplan Meier curves showing breast cancer specific survival (BCSS) in the training set (**D**), disease free survival (DFS) in the training set (**E**), breast cancer specific survival (BCSS) in the test set (**F**) and disease free survival (DFS) in the test set (**G**). See text for details.

Figure 3: SMUG1 protein expression in breast cancer**.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in ER + tumours that received endocrine therapy (training set) (**A**), disease free survival (DFS) in ER + tumours that received endocrine therapy (training set) (**B**), breast cancer specific survival (BCSS) in ER + tumours that received endocrine therapy (test set) (**C**), disease free survival (DFS) in ER + tumours that received endocrine therapy (test set) (**D**), breast cancer specific survival (BCSS) in ER - tumours that received chemotherapy (**E**), disease free survival (DFS) in ER - tumours that received chemotherapy (**F**). See text for details.

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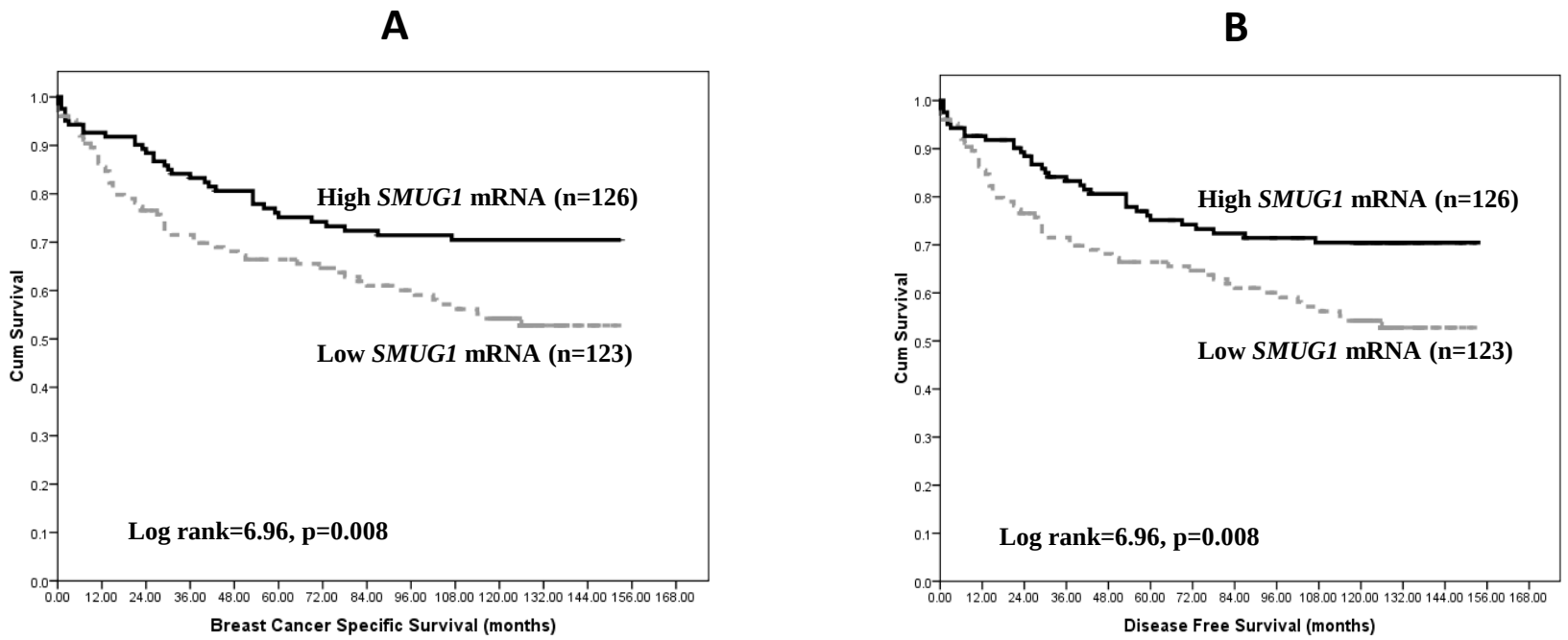

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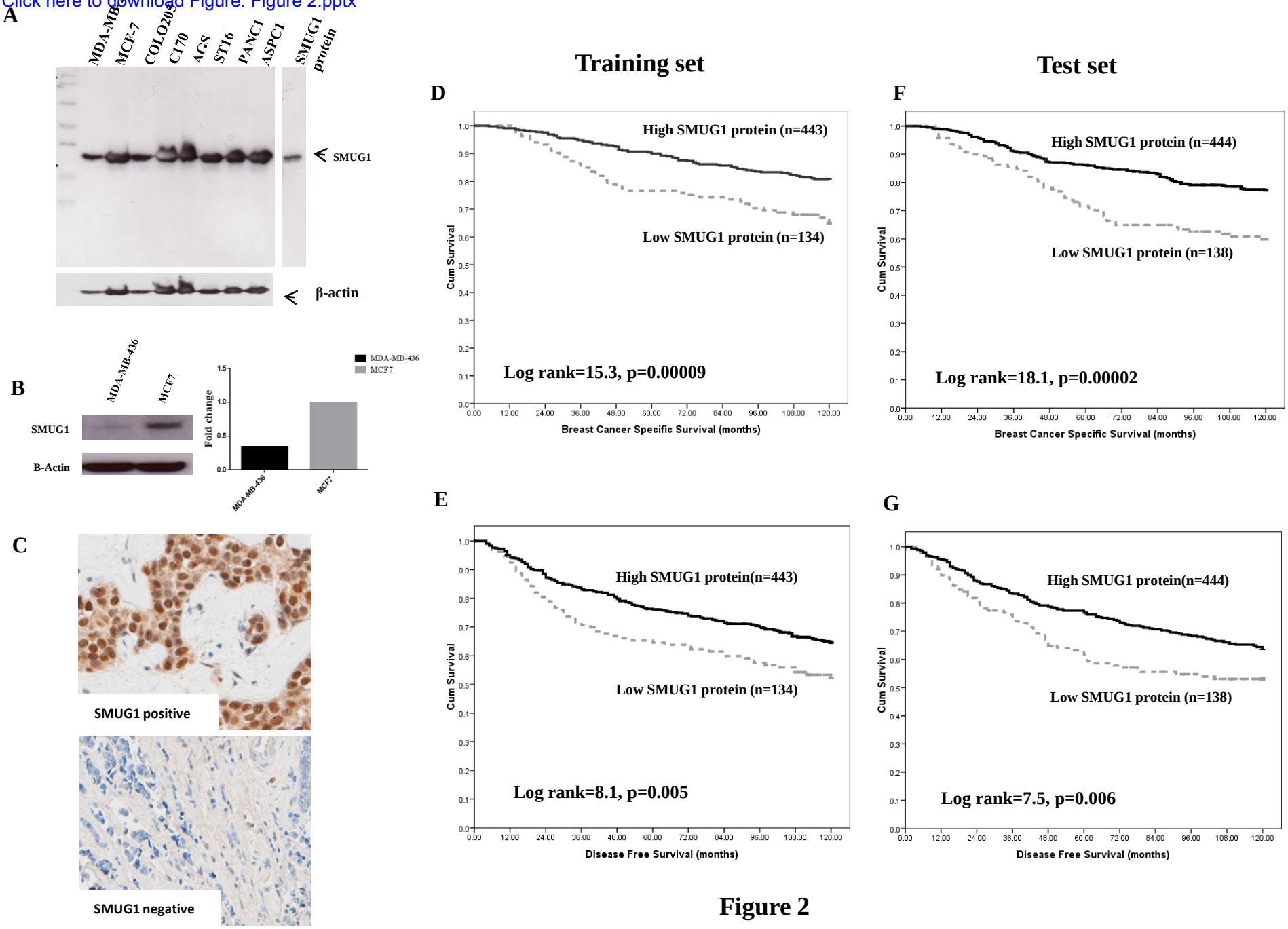

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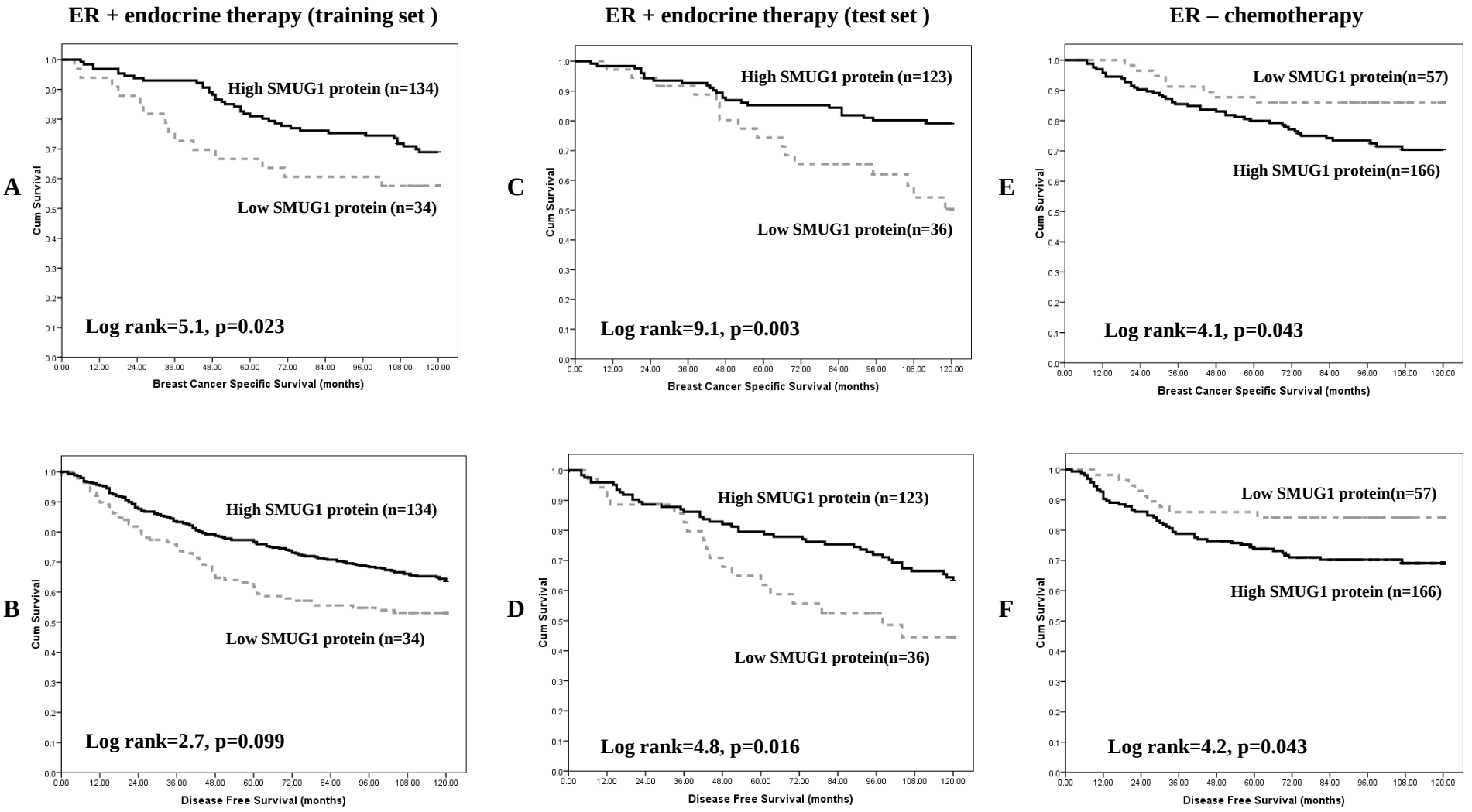

Figure 3

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