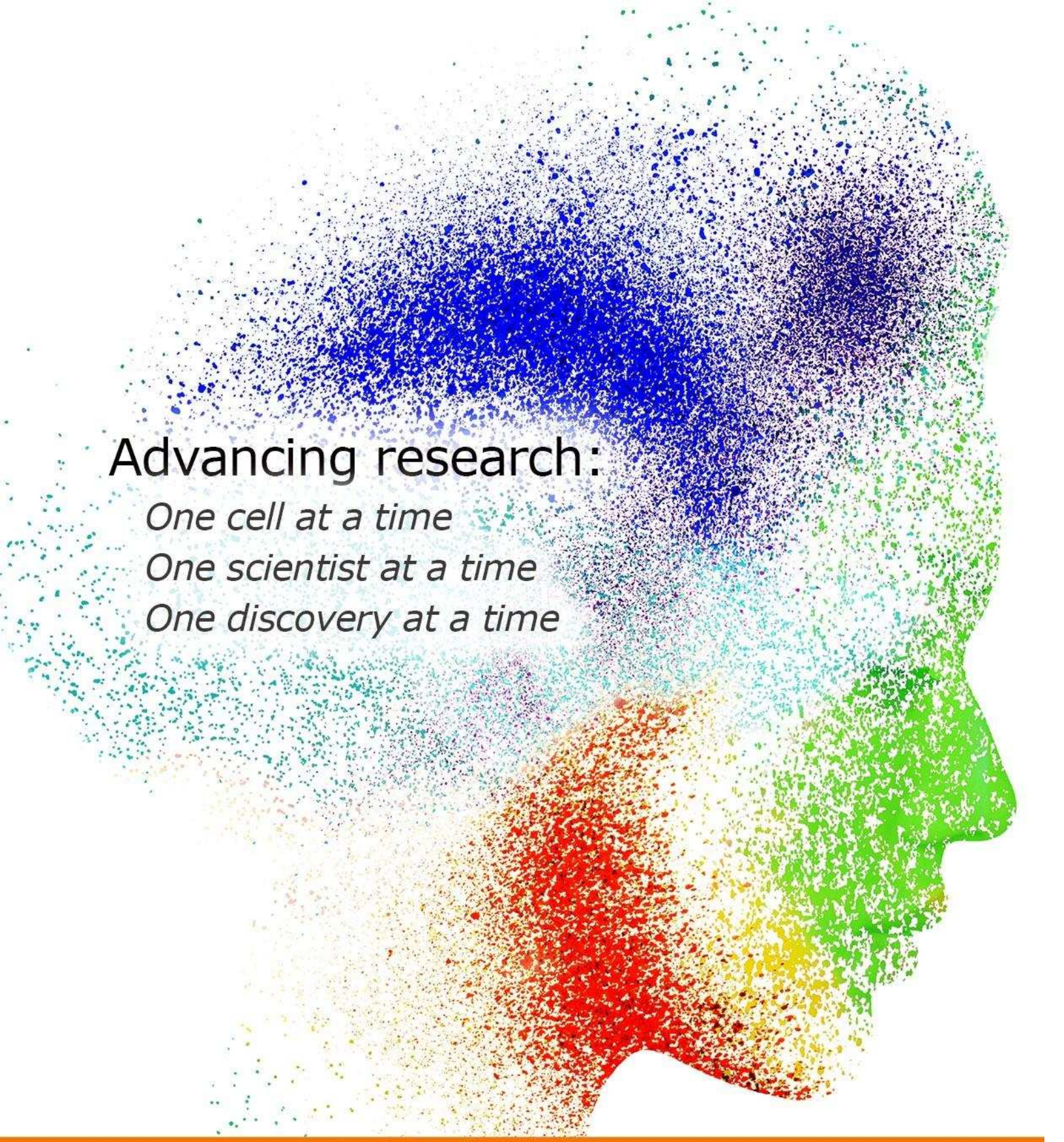

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Expression of miRNA-26b-5p and its target TRPS1 is associated with radiation exposure in post-Chernobyl breast cancer

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Novelty and Impact Statement

Ionising radiation is known to be a risk factor for the development of breast cancer. We identified two markers specific for radiation-associated breast cancer in a cohort of female post-Chernobyl clean-up workers. The markers have the potential to allow identification of radiation-induced breast cancer and thereby to provide the basis for individualised therapy. Further these findings pave the ground for the investigation of the molecular mechanisms of radiation-induced breast cancer.

Abstract

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Ionising radiation is a well-recognised risk factor for the development of breast cancer, however, it is unknown whether radiation-specific molecular oncogenic mechanisms exist. We investigated post-Chernobyl breast cancers from radiation-exposed female clean-up workers and non-exposed controls for molecular changes. Radiation-associated alterations identified in the discovery cohort (n=38) were subsequently validated in a second cohort (n=39). Increased expression of hsa-miR-26b-5p was associated with radiation exposure in both of the cohorts. Moreover, downregulation of the TRPS1 protein, which is a transcriptional target of hsa-miR-26b-5p was associated with radiation exposure. Since TRPS1 overexpression is common in sporadic breast cancer its observed downregulation in radiation-associated breast cancer warrants clarification of the specific functional role of TRPS1 in the radiation context. For this purpose, the impact of TRPS1 on the transcriptome was characterised in two radiation-transformed breast cell culture models after siRNA-knockdown. Deregulated genes upon TRPS1 knockdown were associated with DNA-repair, cell cycle, mitosis, cell migration, angiogenesis and EMT pathways. Furthermore, we identified the interaction partners of TRPS1 from the transcriptomic correlation networks derived from gene expression data on radiation-transformed breast cell culture models and sporadic breast cancer tissues provided by the TCGA database. The genes correlating with TRPS1 in the radiation-transformed breast cell lines were primarily linked to DNA damage response and chromosome segregation, whilst the transcriptional interaction partners in the sporadic breast cancers were mostly associated with apoptosis. Thus, upregulation of hsa-miR-26b-5p and downregulation of TRPS1 in radiation-associated breast cancer tissue samples suggests these molecules representing radiation markers in breast cancer.

Keywords

TRPS1, hsa-miR-26b-5p, Chernobyl, breast cancer, radiation-associated

Introduction

Breast cancer is one of the most common cancers in women worldwide. Beside risk factors such as age and lifestyle it is well-recognised that breast cancer risk increases with exposure to ionising radiation. Patients with preceding radiotherapy for the treatment of Hodgkin lymphoma exhibit an increased risk to develop breast

cancer as a secondary tumour. In the Japanese atomic bomb survivors cohort a similar finding has been reported for women who were exposed to ionising radiation during adolescence.[1] Also in the aftermath of the Chernobyl accident in 1986 a significant increase of the breast carcinoma rate (standardised incidence ratio 190.6%) in female clean-up workers was noticed in comparison to sporadic breast cancer rates in Ukraine.[2, 3] To date, despite this epidemiologic evidence the knowledge about radiation-specific mechanisms of breast carcinogenesis after low-dose exposure is sparse.

In contrast to environmental exposures of patients from the present study, aberrant expressions of miRNAs after exposure to therapeutic doses of ionising radiation have already been reported.[4] miRNAs are 19-25 nucleotides long, non-coding, highly conserved RNA molecules, that are known to play an important role in the regulation of gene expression at the post-transcriptional level. Numerous studies have shown a deregulation of miRNAs in tumours, thereby demonstrating that miRNAs are involved in the process of carcinogenesis and act as oncogenes or as tumour suppressors.[5] Breast cancer-specific miRNA profiles have been observed for different breast cancer subtypes, enabling a classification into different molecular subtypes.[6] However, the role of miRNAs in radiation-associated breast cancer has not been investigated so far. Therefore, our study intended to investigate the miRNA profiles of breast cancers from a cohort of female clean-up workers who were exposed to ionising radiation from the Chernobyl reactor accident and non-exposed controls matched for residence, tumour type, age at diagnosis, TNM-classification and grading. We explored if among miRNAs that are known to play a role in sporadic breast cancer there are specifically radiation-associated ones. We discovered that expression of hsa-miR-26b-5p was increased in radiation-associated breast cancers compared to non-exposed controls. Further, we showed that expression of one of the hsa-miR-26b-5p target proteins TRPS1 (Trichorhinophalangeal syndrome type 1) was significantly decreased in radiation-exposed cases. TRPS1 is a GATA-type transcription factor and consists of nine zinc-finger domains, including a single GATA-type DNA-binding domain. Either mutation or deletion of this gene causes a disease called Trichorhinophalangeal syndrome. Previous studies have shown that TRPS1 is expressed in several human malignant tumours and implied an important function in tumour growth, cell cycle, angiogenesis, apoptosis, cell proliferation, migration and metastasis.[7-13]

In this study, we were able to identify for the first time one miRNA and one of its target proteins to be significantly associated with radiation-associated breast cancer.

Material and Methods

Patients tumour tissues and clinical data

Formalin-fixed paraffin-embedded (FFPE) breast cancer tissue samples (n=76) from 38 female Ukrainian patients that were exposed to radiation after the Chernobyl reactor accident and a matched set of 38 breast cancer samples from non-exposed patients from Ukraine were collected (discovery set). The vast majority (34 out of 38) of patients have been exposed as clean-up workers after the accident for which an elevated breast cancer incidence has been reported.[2, 3] Four patients were exposed as evacuees after the accident. The mean age at time of exposure was 33 years (range 18-45 years), the mean age at time of diagnosis was 49 years (range 33-59 years), and the mean latency of tumours was 16 years. None of the patients from the discovery set received neoadjuvant radio(chemo)therapy (Table 1).

A validation cohort consisting of FFPE breast cancer tissue samples, 39 from post-Chernobyl clean-up workers and 39 matched non-exposed Ukrainian control cases, was established. The mean age at time of exposure was 33 years (range 23-40 years) and the mean age at diagnosis 50 years (range 35-59 years) and the mean latency was 18 years. 18 out of 78 patients of the validation cohort received a neoadjuvant radio(chemo)therapy (Table 1).

The so called RADRUE method, which was adapted specifically for estimation of breast doses, was used for reconstruction of the absorbed doses of the exposed breast cancer patients.[14] Information about the absorbed doses were only available for a subset of the exposed breast cancer patients (n=54). The absorbed doses showed a large inter-individual variability between patients ranging from 0.06 to 929 mGy (median 8.53 mGy) in the clean-up workers and from 5.72 to 36.85 mGy (median 18.68 mGy) in the evacuees. (unpublished data)

In both cohorts, all patients were younger than 60 years at the time of diagnosis. Exposed patients and non-exposed controls for this case-case study were frequency matched for residence, tumour subtype, age at diagnosis, TNM-classification and histological grading. The majority of tumours was diagnosed as invasive carcinoma

of no special type (NST; discovery cohort: 95 %, validation cohort: 90 %) and invasive lobular carcinoma (ILC; discovery cohort: 2.5 %, validation cohort: 8 %). Two cases were diagnosed as intracystic papillary breast carcinoma and another two as breast carcinomas with medullary features. Immunohistochemical staining for estrogen and progesteron receptors, C-kit, Cytokeratin 5/6, TP53 and ki67 antigen expression as well as HER2 gene status determination by Fluorescence *in situ* Hybridization (FISH) is described in the supplementary material and methods part.

Information of all clinicopathologic characteristics of the discovery and validation cohort is presented in Table S1 and S2 in the supplementary information part.

Total RNA including the small RNA fraction was isolated using the Qiagen RNeasy FFPE Kit (Qiagen, Hilden, Germany). Small RNA (miRNA) integrity was analysed by qRT-PCR of the small non-coding RNA RNU24 using TaqMan chemistry (Life technologies, Carlsbad, CA, USA). Samples with Ct values < 35 were considered suitable for analysis.

Fisher's exact test was used to test associations of the exposure status with any clinical characteristics of the patients such as estrogen-receptor status, progesterone-receptor status, cytokeratin-expression status (positive/negative), C-kit-expression status (positive/negative), Ki67-expression status (positive/negative), Her2/neu-status, p53-mutation status status, BRCA1/2-mutation status, pT-status, pN-status and grading. Significance was accepted for $p < 0.05$.

Quantitative realtime RT-PCR (qRT-PCR)

Reverse transcription of miRNAs was performed using the MicroRNA Reverse Transcription Kit and microRNA-specific stem-loop primers according to the manufacturer's protocol (Life Technologies). TaqMan MicroRNA assays (Life Technologies) for the following miRNAs were used: *hsa-miR-222-3p* (477982_mir), *hsa-miR-221-3p* (477981_mir), *hsa-miR-372-3p* (478071_mir), *hsa-miR-26b-5p* (478418_mir), *hsa-miR-302d-3p* (478237_mir), *hsa-miR-124-3p* (477879_mir), *hsa-miR-1-3p* (477820_mir) and *hsa-miR-99b-5p* (478343_mir). For endogenous normalisation the assays for *RNU44* (001094) and *RNU48* (001006) were used. qRT-PCR reactions (20 µl) were carried out in triplicates using the ViiA 7 Real Time PCR System in combination with the ViiA 7 Software v.1.2.2 following the manufacturer's protocol (Life Technologies). Relative expressions were calculated using the ΔΔCt method. The partial differential test considering inter-tumour heterogeneity was used

to test for statistical significant differences of miRNA expressions between exposed and non-exposed samples and possible associations of miRNA-expression with clinicopathological data.[15]

TRPS1 (Hs00232645_m1) TaqMan gene expression assay (LifeTechnologies) was used to validate the TRPS1-knockdown and to determine the TRPS1-knockdown efficacy in B42-11 and B42-16 cells at gene expression level.

For technical validation of the gene expression microarray data qRT-PCR was performed for randomly selected genes (n=12) detected by gene expression microarray in B42-11 and B42-16 cells: *ANXA1* (Hs00167549_m1), *APRT* (Hs00975725_m1), *BBC3* (Hs00248075_m1), *BMP2* (Hs01055564_m1), *CLNS1A* (Hs00818054_m1), *DTL* (Hs00978565_m1), *DUSP6* (Hs00169257_m1), *F2R* (Hs00169258_m1), *PLK2* (Hs01573405_g1), *RFC5* (Hs00738859_m1), *TRPS1* (Hs00232645_m1) and TUBB3 (Hs00801390_s1). For endogenous normalisation the assays for *ACTB* (Hs99999903_m1) and *B2M* (Hs99999907_m1) were used. RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR reactions (10 µl) and calculations of relative expressions were carried out as described above. For technical validation of the gene expression microarray data, Pearson correlation analyses of expression determined by qRT-PCR with that determined by microarray were performed. Validation was considered successful for correlation coefficients > 0.5 and p-values < 0.05.

Immunohistochemistry

The expression of the TRPS1 protein in both tumour cohorts was measured by immunohistochemical staining (IHC) of FFPE tumour sections with a primary antibody against TRPS1 (Abcam: ab111439, Cambridge, UK). The antibody was selected from Abcam with information about antibody specificity and staining patterns.[9] The primary antibody was used in a dilution of 1:100 and Discovery-Universal (Roche, Ventana, Tucson, AZ, USA) as a secondary antibody. IHC staining was performed with the automated staining instrument Discovery XT (Roche, Ventana) system using peroxidase-DAB-(diaminobenzidine)-MAP chemistry (Roche, Ventana) for signal detection. The stained tissue sections were fixed in an ethanol series and coated by a coverslip. All stained slides were scanned at 20x objective magnification using the Leica SCN400 digital slide scanning system (Leica, Houston, USA).

Digital image analysis

The evaluation of the immunohistochemical staining was performed using the digital image analysis platform DefiniensTissueStudio 3.5 (Definiens AG; Munich, Germany). For this purpose the digital slide images were imported into the image analysis software. In a first step regions of interest, that is tumour area, were manually defined. A specific rule set was then created in order to detect and quantify the TRPS1 stained nuclei within the annotated tissue areas. The quantified parameters were the amount and the mean brown intensity of TRPS1 positive nuclei per annotated tissue area. The averaged TRPS1 staining intensities were tested for significant differences between exposed and non-exposed samples and possible associations of TRPS1 staining intensities with clinicopathological data using partial differential testing, which considers inter-tumour heterogeneity.[15] P-values smaller than 0.05 were considered statistically significant.

B42-11 and B42-16 cell lines and spectral karyotyping (SKY)

Human B42-11 and B42-16 radiation transformed breast cells were grown in mammary epithelial growth medium (MEGM) as published previously.[16] The B42-11 and B42-16 cell line were authenticated by STR-typing and spectral karyotyping (SKY). Metaphase chromosome spreads were prepared and hybridised as described earlier.[17] SKY image analysis was performed with a SpectraCube system and SkyView imaging software (Applied Spectral Imaging).

RNA interference

The B42-11 and B42-16 cells were seeded into six-well plates and were transfected at 70-90 % confluency in triplicates with a non-sense scrambled control (Ambion, Carlsbad, CA, USA; Negative control #1) or two specific siRNAs against TRPS1 (Ambion, silencer select siRNA 1: ID: s14428 and siRNA 2: ID: s14427). SiRNA transfections were performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. 7.5 µl Lipofectamine and 3.75 µl of TRPS1 siRNA were used per sample resulting in a siRNA concentration of 75 pmol per well. After 24 h, 48 h, 72 h and 96 h cells were harvested for total RNA isolation using the Qiagen RNeasy Mini Kit (Qiagen). In addition, protein lysates were generated 48 h and 72 h after transfection to verify TRPS1-knockdown efficacy by Western Blot analysis.

Western blot analysis

Western blot analysis with an antibody against TRPS1 (Abcam: ab111439) was performed in order to monitor the TRPS1 knockdown at protein level. RIPA-buffer (150 mM NaCl, 1% NP-40, 10 mM MDOC, 0.1% SDS, 50 mM Tris pH 8.0 supplemented with protease, phosphatase and HDAC inhibitors) was used for protein extraction and was performed on ice. 25 µg of total protein was used for each Western blot analysis. The proteins were separated on a 10 % SDS-PAGE. PVDF-membranes were cut and blocked with 8 % skim milk buffer after immunoblotting followed by incubation over night at 4°C with primary antibodies (rabbit polyclonal anti-TRPS1, Abcam: ab111439; 1:2000; mouse monoclonal anti-ß-Actin, Sigma: A5441; 1:10000) diluted in Roti-Block (Roth). After four washing steps with TBST-buffer (5 min each), the PVDF-membranes were incubated for 2 hours with a secondary antibody (anti-rabbit IgG, Jackson ImmunoResearch; 1:50000, anti-mouse IgG Jackson ImmunoResearch; 1:50000), diluted in 8 % skim milk buffer. Blots were developed with Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, United Kingdom). Chemiluminescence was detected and images were acquired with a FluorChem HD2 documentation system from Alpha Innotech in combination with the AlphaView software (Biozym, Oldendorf, Germany).

Microarray-based gene expression analysis

In order to investigate the effect of TRPS1-knockdown on the transcriptome, mRNA microarray expression profiling of biological triplicates of cells after TRPS1-knockdown, a non-sense scrambled control and the B42-11 and B42-16 untreated cell lines 48 h after transfection was performed using G3 Human Gene Expression 8x60k v2 microarrays (AMADID 72363, Agilent Technologies, Santa Clara, CA). RNA quality was assessed prior to expression analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies). The obtained RNA integrity numbers (RINs) ranged from 6.7 to 9.7. The analysis was performed according to the manufacturer's instructions using 50 ng of total RNA. Microarrays were scanned using a G2505C Sure Scan Microarray Scanner (Agilent Technologies) followed by raw data extraction with the Feature Extraction 10.7 software (Agilent Technologies). Data quality assessment, pre-processing, and normalisation were conducted in R using the Bioconductor AgiMicroRNA package.[18] Statistical analyses were performed using functions from the Bioconductor limma package for the identification of significantly differentially

expressed genes after TRPS1-knockdown (siRNA 1 and siRNA 2 taken together) compared to the non-sense scrambled control.[19] A cut-off for FDR-adjusted p-values of 0.05 and minimum absolute log2-fold change of 0.5 was applied. Significantly deregulated genes after TRPS1 knockdown were subjected to pathway enrichment analysis using the Cytoscape Reactome Functional Interaction (FI) plugin (version 2016) within the Cytoscape network visualisation software (version 3.5.1).[20, 21] For pathway enrichment analysis, only network modules containing more than three genes were considered. The top 50 pathways with a FDR-adjusted p-value < 0.05 were considerered for further interpretation.

TRPS1-centred correlation network

In order to explore potential direct and indirect interaction partners of TRPS1 at the transcriptome level we generated gene correlation networks from the microarray gene expression data on B42-11 and B42-16 untransfected, scrambled-siRNA transfected and TRPS1-downregulated cells and from global mRNA expression data on sporadic breast cancers of the publicly available The Cancer Genome Atlas (TCGA) breast cancer dataset.[22, 23] The latter of which were matched to the breast cancer post-Chernobyl cohort for the parameters tumour type, hormone receptor status, age, TNM-classification, grading, BRCA1/2- and Her2-status. For both data sets correlation (Pearson) of the TRPS1 expression vector and all other genes was determined and a correlation test was applied. The resulting p-values were corrected for multiple-testing error determining the Benjamini-Hochberg FDR.[24] A cut-off for FDR-adjusted p-values of 0.05 was applied. The top 100 correlating genes were selected and subjected to GO-term and pathway enrichment analysis using the ClueGo plugin (version 2.3.2, 2016) of the Cytoscape network analysis software (version 3.0.2).[21, 25] The top 50 pathways with a FDR-adjusted *p*-value < 0.05 were considerered for further interpretation.

Results

Selection of candidate miRNAs

We explored the literature by PubMed research and identified the following miRNAs to be most frequently published as being associated with breast cancer and radiation exposure: hsa-miR-26b-5p, hsa-miR-99b-5p, hsa-miR-221-3p and hsa-miR-222-3p.[13, 26-29] Commonly regulated target genes of these miRNAs were identified using

MiRTarBase (version 4.0, 2014) and revealed the gene TRPS1 (The trichorhinophalangeal syndrome 1).[30] According to MiRTarBase (version 4.0, 2014) TRPS1 is regulated by additional four miRNAs: hsa-miR-124-3p, hsa-miR-302d-3p, hsa-miR-1-3p and hsa-miR-372. We selected these eight TRPS1-regulating miRNAs and the target protein TRPS1 for further analysis in the discovery and validation cohorts.

Increased hsa-miR-26b-5p expression is associated with radiation exposure

The analysis of the selected miRNAs was performed by qRT-PCR and subsequent partial differential testing between the exposed and non-exposed tumour sets. Hsa-miR-221-3p (FC=1.87, partial differential test p-value=0.006), hsa-miR-222-3p (FC=1.39, partial differential test p-value=0.03) and hsa-miR-26b-5p (FC=1.97, partial differential test p-value=0.01) were significantly upregulated in the exposed compared to the non-exposed tumour set of the discovery cohort. The other miRNAs did not show statistically significant deregulation between exposed cases and controls. From the three miRNAs that were found to be significantly associated with radiation exposure in the discovery cohort upregulation of hsa-miR-26b-5p could be confirmed in the exposed cases of the validation cohort (FC=1.3, p=0.02, Figure 1A/B). Hsa-miR-26b-5p expression was not associated with estrogen-receptor status, progesteron-receptor status, cytokeratin-expression (positive/negative), c-kit-expression (positive/negative), Ki67-expression (positive/negative), Her2/neu-status, TP53-status and BRCA1/2-mutation status in the discovery or the validation cohort. Moreover, no dose-response effect was observed for hsa-miR-26b-5p (data not shown). We also tested if the exposure status was associated with any clinical characteristics of the patients, whereby no significant association between exposure status and any of the clinical characteristics could be detected (Table 1).

Decreased TRPS1 protein expression is associated with radiation exposure

The expression of the TRPS1 protein, which was identified as a target of the literature-derived candidate miRNAs, was determined by immunohistochemical staining of serial FFPE tissue sections and subsequently tested for association with radiation exposure. After software-based quantification of staining intensities a significant downregulation of TRPS1 protein expression in breast cancer tissues from exposed patients was detected (p=0.028). This finding was confirmed in the

validation cohort (p=0.027). Visualisation of these results can be found in Figure 2 and Figures 3A-B. Further, no dose-response effect was observed for TRPS1 (data not shown).

Association of TRPS1 expression with clinical and histological data

For all tumour samples of the discovery and validation cohorts an association of the TRPS1 protein expression with other clinical parameters was tested (partial differential test). TRPS1 protein expression was not associated with estrogen-receptor status, progesterone-receptor status, cytokeratin-expression (positive/negative), c-kit-expression (positive/negative), Ki67-expression (positive/negative), Her2/neu-status, TP53-status and BRCA1/2-mutation status in the discovery and the validation cohort, suggesting an independent association of TRPS1 downregulation with radiation exposure of patients.

Characterisation of the B42-11 and B42-16 cell lines

SKY analysis revealed the following karyotype for B42-16 resulting from evaluation of 15 metaphases: 47,XX,der(4)t(4;12)(p31;?),i(8)(q10),+der(8)t(8;10)(q21;?),der(10)t(8;10;12)(?;p12;q23;?),der(12)t(8;10:12)(?;?;q22) and for B42-11: 47,XX, +i(8)(q10),der(7)t(7;10)(q11.1;11.2). A representative metaphase for each is shown in Figure S1.

TRPS1 knockdown in B42-11 and B42-16 cells

In order to characterise the impact of TRPS1 on the transcriptome in radiation-transformed breast cells, siRNA-knockdown of TRPS1 was performed in the radiation-transformed breast cell lines B42-11 and B42-16. The knockdown reached a maximum after 48h (Figure 4, Figure S2), therefore this timepoint was chosen for differential expression analysis between TRPS1-knockdown and scrambled control of B42-11 and B42-16 cells. The analysis revealed 281 significantly differentially expressed microarray probes (144 down- and 137 up-regulated) relating to 267 different genes (Table S3). Randomly selected genes (n=12) detected by gene expression microarray in B42-11 and B42-16 cells were chosen for technical validation of the microarray data. Correlation analysis of expression of the genes selected for validation determined by qRT-PCR and mRNA microarray showed

strong correlation for ten out of 12 analysed genes (Table S4). Further, pathway enrichment analysis was conducted based on the Reactome network, resulting in nine modules containing the 267 significantly deregulated genes after TRPS1 knockdown. Significantly enriched pathways involving DNA-repair, cell cycle, mitosis, cell migration, angiogenesis and EMT were detected (Table S5). Downregulated genes were mainly involved in DNA-repair, cell cycle and mitosis whilst upregulated genes mostly showed up in cell migration, angiogenesis and EMT pathways (Table S5).

TRPS1-centred correlation network

In order to explore putative direct and indirect interaction partners of TRPS1 in the sporadic and radiation-associated context at the transcriptome level, two TRPS1-centred correlation networks were generated and subsequently analysed for involved pathways. To examine the role of TRPS1 in sporadic breast cancer, we deployed the RNAseq derived global gene expression data set on breast cancer from the The Cancer Genome Atlas (TCGA) dataset.[22, 23] From the 1106 available cases a subset that matched our radiation-associated breast cancer cohort (n=382) was used. In total, 12106 genes showed a statistical significant correlation with TRPS1 expression in sporadic breast cancers of the publicly available TCGA dataset and 1270 genes in the B42-11 and B42-16 cells (FDR < 0.05) (Table S6).

From both correlation networks we selected the top 100 correlating genes with regard to FDR (Figure 5A/B, Table S6). GO and pathway enrichment analysis including the top 100 correlating genes of the sporadic breast cancer correlation network revealed mainly significant enrichment of apoptosis related pathways such as *TRADD:TRAF2:RIP1 complex binds FADD* and *RIPK1 is deubiquitinated*. The radiation-associated cell lines B42-11 and B42-16 showed mainly significant enrichment of GO-terms related to the process of chromosome segregation and DNA-repair.

Discussion

Radiation-specific markers have already been reported in young patients suffering from papillary thyroid carcinomas in the aftermath of the Chernobyl accident.[31] Although ionising radiation is also known to be a risk factor for the development of breast cancer, radiation-specific markers in these tumours are still unknown.[2, 3, 32]

The present study aimed for the discovery of radiation-specific changes of miRNA and protein expressions in breast cancer samples from Ukrainian clean-up workers, who were exposed to ionising radiation from the Chernobyl accident by comparison with non-exposed Ukrainian control cases matched for age and clinical parameters.

From the published literature we identified four miRNAs (hsa-miR-26b-5p, hsa-miR-99b-5p, hsa-miR-221-3p and hsa-miR-222-3p) that were associated with breast cancer and radiation exposure.[13, 26-29] The TRPS1 gene was recognised as a common target gene that is regulated by additional four miRNAs (hsa-miR-124-3p, hsa-miR-302d-3p, hsa-miR-1-3p and hsa-miR-372).[30] The eight TRPS1-regulating miRNAs in total along with the TRPS1 protein were investigated on two independent post-Chernobyl breast cancer cohorts from clean-up workers. Consistently, a significant upregulation of hsa-miR-26b-5p in exposed compared to matched non-exposed patients became aparent in both cohorts and thus, an association of hsa-miR-26b-5p with radiation exposure could be validated independently (Figure 1). Hsa-miR-26b-5p plays a pivotal role in sporadic breast cancer.[29] In sporadic breast cancer decreased hsa-miR-26b-5p expression was reported, and could be confirmed in our sporadic breast cancer control cases. Hsa-miR-26b-5p obviously plays a tumour-suppressive role by the promotion of apoptosis and the suppression of cell growth.[29, 33] An opposed observation in post-Chernobyl cases points to a radiation-specific deregulation of hsa-miR-26b-5p and renders the question whether TRPS1 is also affected. Surprisingly, also the TRPS1 expression was significantly downregulated in both exposed breast cancer cohorts compared to the non-exposed cohorts. Since this finding was confirmed in two independent cohorts it suggests an important role of TRPS1 in radiation-associated breast cancer (Figures 2, 3). To our knowledge TRPS1 and hsa-miR-26b-5p alterations have not been investigated in radiation-associated breast cancers so far. In sporadic breast cancer an upregulated TRPS1 expression was previously reported which is in line with our findings in the sporadic subset of control cases.[34] In sporadic breast cancer TRPS1 is linked to the stimulation of cell proliferation as well as angiogenesis and the promotion of cell cycle progression.[7, 10, 12] Furthermore, TRPS1 overexpression was proposed as a prognostic marker in early stage breast cancer due to an association with improved overall survival and disease-free survival in these tumours.[35] Moreover, TRPS1 expression was found to correlate with ER, PgR, Ki67, GATA-3 and Her2 expression, which we could not confirm in our data.[36, 37] At the same time TRPS1 acts as a

negative regulator of EMT and thus could reduce the metastatic potential of breast cancers by suppressing transcriptionally the processes of migration and invasion.[11, 13] Taken together the published data on TRPS1 overexpression in sporadic breast cancer and its impact on tumour progression suggests in turn a more aggressive tumour behaviour in radiation-associated breast cancers with downregulated TRPS1.

To clarify the functional consequences of TRPS1 downregulation in the radiation-associated context, we performed siRNA-knockdown experiments in radiation transformed breast cells B42-11 and B42-16. A time-course analysis of TRPS1 expression after siRNA-transfection (Figure S2) showed a downregulation of TRPS1 compared to the scrambled control at the mRNA and protein levels (Figure 4). The major goal of this knockdown experiment was to establish a gene-correlation network in radiation-associated B42-11 and B42-16 cells based on global transcriptomic data for functional insights. A pathway enrichment analysis of differentially expressed genes revealed a significant enrichment of pathways related to DNA-repair, cell cycle, mitosis, cell migration, angiogenesis and EMT (Table S5). This is in good agreement with the expectations from the published data as discussed above. However, a novel finding of this study is the effect of TRPS1 downregulation on DNA-repair pathways in radiation-associated B42-11 and B42-16 cells pointing to radiation-induced effects in these cells. Furthermore, gene-expression-microarray data could be technically validated by qRT-PCR (Table S4).

The gene interaction network of TRPS1 from global transcriptomic data of the TRPS1-knockdown in B42-11 and B42-16 cells was compared to a TRPS1-centred correlation network based on global mRNA expression data from matched sporadic breast cancers of the publicly available TCGA dataset (Figure 5A/B, Table S6). The main difference between both networks was a significant enrichment of apoptosis-related processes in sporadic tumours, whilst a link to DNA-repair, chromosome segregation and genomic instability became apparent in the radiation transformed cell lines B42-11 and B42-16 (Table S7). The involvement of TRPS1 in chromosome segregation has already been described in chondrocytes.[38] Many of the top ten genes interacting with TRPS1 are known to be involved in fundamental carcinogenic processes such as DNA-repair and cell migration. For example, GPR64 and LYAR (TRPS1-interaction partners in B42-11 and B42-16 cells showing a positive correlation with TRPS1) are known to be involved in the process of migration. GPR64 is known to be involved in the adhesion and migration of breast cancer cells

through mechanisms including a non-canonical NFkB pathway.[39] Furthermore, it was reported that transcription factor LYAR promote tumour cell migration and invasion by upregulating galectin-1 gene expression in colorectal cancer.[40] Another interesting network link was RFC5 (activated by TRPS1 in B42-11 and B42-16) since it appeared in many (10 out of 14) pathways related to DNA-repair and cell cycle/mitosis from the differential expression analysis and is among the top five genes correlating with TRPS1. The RFC5 gene belongs to the replication factor C family and was described to reflect the hallmark of cancer "genomic instability".[41] It was already reported that RFC5 recognize DNA damage and is involved in pathways related to the process of mismatch repair.[42, 43] Furthermore, an aberrant expression of this gene was already observed in several tumour entities.[42, 44, 45]

This suggests deregulation of cellular processes involved in radiation-induced damage response. In all, there are several hints that TRPS1 plays a specific role in DNA-repair, chromosome segregation and genomic instability which is a well-established phenotype after irradiation and in radiation-associated carcinogenesis.[46] A link of TRPS1-interaction partners to DNA-repair and chromosome segregation is not obvious from the TRPS1-centred correlation network derived from the sporadic breast cancer TCGA dataset suggesting this being a specific effect of TRPS1 deregulation in radiation-associated breast cancer. Moreover, most of the top ten TRPS1-interaction partners derived from the sporadic dataset are known to be involved in apoptosis, cell migration and cell cycle which is in agreement with the published literature on TRPS1 in sporadic breast cancer and prostate cancer.[47-49]

It was already shown in MCF7 breast cancer cells that TRPS1 functions as a transcription activator of FOXA1 and negatively regulates the expression of ZEB2.[11, 13] An interaction of FOXA1 with TRPS1 was also detected in the correlation network of the sporadic TCGA breast cancer dataset (FOXA1, Pearson correlation=0.17, FDR=0.02). The weak but significant correlation could be due to the fact that the TRPS1-interaction network for sporadic breast cancer in this study was developed from mRNA expressions of tumour tissues in contrast to proteomics data from *in vitro* models as published by Huang et al.[11] The negative association of TRPS1 with ZEB2, however, was not detected in our data. It is interesting to note that there is no common gene between the correlation networks of B42-11 and B42-16 cells and of the sporadic TCGA dataset which again points to specific radiation-associated functions of TRPS1.

In conclusion, this study reveals radiation markers in breast carcinogenesis consisting of an upregulated hsa-miR-26b-5p and a downregulation of the validated target protein TRPS1. Both markers could be validated in independent tumour cohorts of radiation-associated post-Chernobyl breast cancers, suggesting an important role in radiation-induced carcinogenesis. Moreover, we could identify interaction partners of TRPS1 in TRPS1-knockdown models that point to a functional role of TRPS1 in radiation-associated breast carcinogenesis in DNA damage response and tumour progression.

Abbreviations

FDR, false discovery rate; FFPE, formalin-fixed paraffin-embedded; GO, gene ontology; IHC, immunohistochemistry; ILC, invasive lobular carcinoma; NST, invasive carcinomas of no special type; PVDF, Polyvinylidenfluorid; SDS, sodium dodecyl sulfate polyacrylamide; TBST Tris-buffered saline Tween20; TNM, primary tumour, lymphnode metastases, distant metastases; TRPS1, Trichorhinophalangeal syndrome type 1; PTC, papillary; SKY, Spectral imaging; qRT-PCR, quantitative real time reverse transcription polymerase chain reaction.

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Tab. 1) Patient characteristics of the Chernobyl discovery and validation cohort

Characteristics		Chernobyl Discovery Cohort			Chernobyl Validation Cohort		
		exposed	not exposed	*P* value[1]	exposed	not exposed	*P* value[1]
Number of patients		38	38		39	39	
Tumour type, no. (%)	invasive carcinoma of no special type	36 (95)	36 (95)	1	35 (90)	35 (90)	1
	lobular	1 (2.5)	1 (2.5)		3 (8)	3 (8)	
	intracystic	0 (0)	0 (0)		1 (2)	1 (2)	
	medullar	1 (2.5)	1 (2.5)		0 (0)	0 (0)	
Estrogen-receptor status, no. (%)	positive	24 (63)	25 (66)	0.89	30 (77)	29 (74)	0.8
	negative	14 (37)	13 (34)		9 (23)	11 (28)	
Progesteron-receptor status, no. (%)	positive	22 (58)	27 (71)	0.34	26 (67)	29 (74)	0.62
	negative	16 (42)	11 (29)		13 (33)	10 (26)	
C-kit status, no. (%)	positive	7 (18)	4 (11)	0.52	2 (5)	3 (8)	1
	negative	31 (82)	34 (89)		37 (95)	36 (92)	
Cytokeratin 5/6 status, no. (%)	positive	7 (18)	5 (13)	0.75	6 (15)	1 (3)	0.11
	negative	31 (82)	33 (87)		33 (85)	38 (97)	
P53 status, no. (%)	positive	13 (34)	15 (39)	0.81	24 (62)	22 (56)	0.82
	negative	25 (66)	23 (61)		15 (38)	17 (44)	
Ki-67 status, no. (%)	positive	31 (82)	32 (84)	1	39 (100)	39 (100)	1
	negative	7 (18)	6 (16)		0 (0)	0 (0)	
BRCA1/2 status, no. (%)	positive	1 (3)	3 (8)	0.36	4 (10)	3 (8)	1
	negative	37 (97)	34 (89)		35 (90)	36 (92)	
	not eveluable	0 (1)	1 (3)		0 (0)	0 (0)	
Her2 status, no. (%)	positive	2 (5)	5 (13)	0.43	7 (18)	7 (18)	1
	negative	36 (95)	33 (87)		28 (72)	32 (82)	
	not eveluable	0 (0)	0 (0)		4 (10)	0 (0)	
pT stage, no. (%)	pT1	21 (55)	20 (53)	0.22	10 (26)	12 (31)	0.71
	pT2	14 (37)	18 (47)		28 (72)	25 (64)	
	pT3	3 (8)	0 (5)		1 (2)	2 (5)	
pN stage, no. (%)	pN0	24 (63)	24 (63)	1	17 (44)	17 (44)	1
	PN1	13 (34)	14 (37)		18 (46)	19 (49)	
	pN2	1 (3)	0 (0)		2 (5)	3 (7)	
	pN3	0 (0)	0 (0)		1 (2.5)	0 (0)	
	pNx	0 (0)	0 (0)		1 (2.5)	0 (0)	
pM stage, no. (%)	M0	38 (100)	38 (100)	1	39 (100)	39 (100)	1
Grade, no. (%)	G1	1 (3)	1 (3)	1	2 (5)	2 (5)	1
	G2	24 (63)	24 (63)		26 (67)	26 (67)	
	G3	13 (34)	13 (34)		11 (28)	11 (28)	

[1] The *P values* were calculated by Fisher's-exact test

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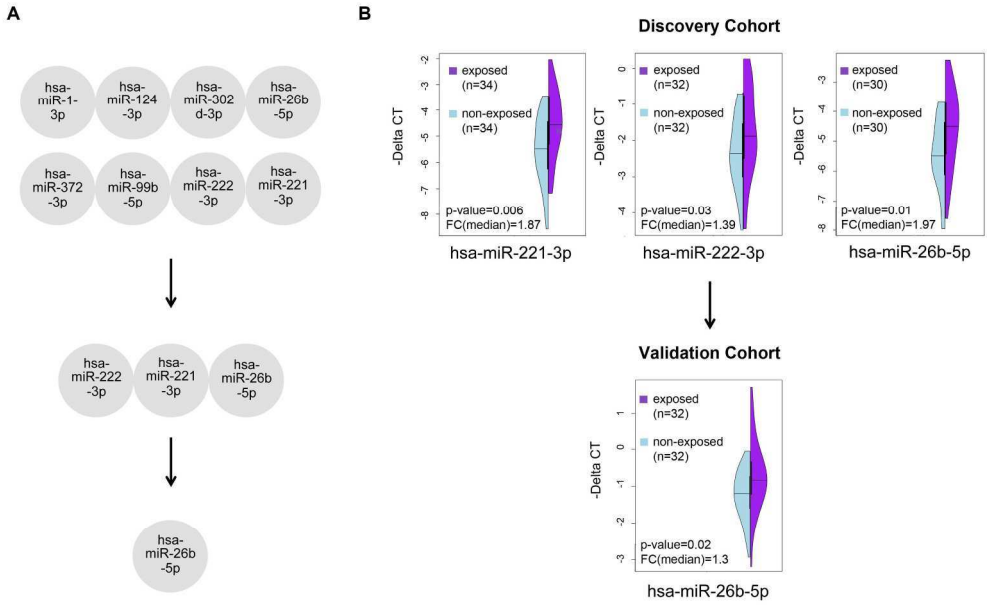

Fig. 1) A: The expression levels of all eight TRPS1-regulating miRNAs were analysed in the Chernobyl discovery cohort by qRT-PCR. Hsa-miR-222-3p, hsa-miR-221-3p and hsa-miR-26b-5p showed a significant differential expression between exposed and non-exposed samples. The expression levels of these three microRNAs were also tested in the Chernobyl validation cohort. The expression of hsa-miR-26b-5p was associated with exposure to ionising radiation in the validation cohort.
B: Violin plots displaying the expressions of hsa-miR-26b-5p, hsa-miR-221-3p and hsa-miR-222-3p in the Chernobyl discovery cohort and hsa-miR-26b-5p in the Chernobyl validation cohort measured by qRT-PCR (-ΔCT-values) are shown (right panel). The non-exposed control group is labeled in light blue, the exposed group in purple. The middle dark line represents the median of expression values. The vertical black line represents the interquartile.

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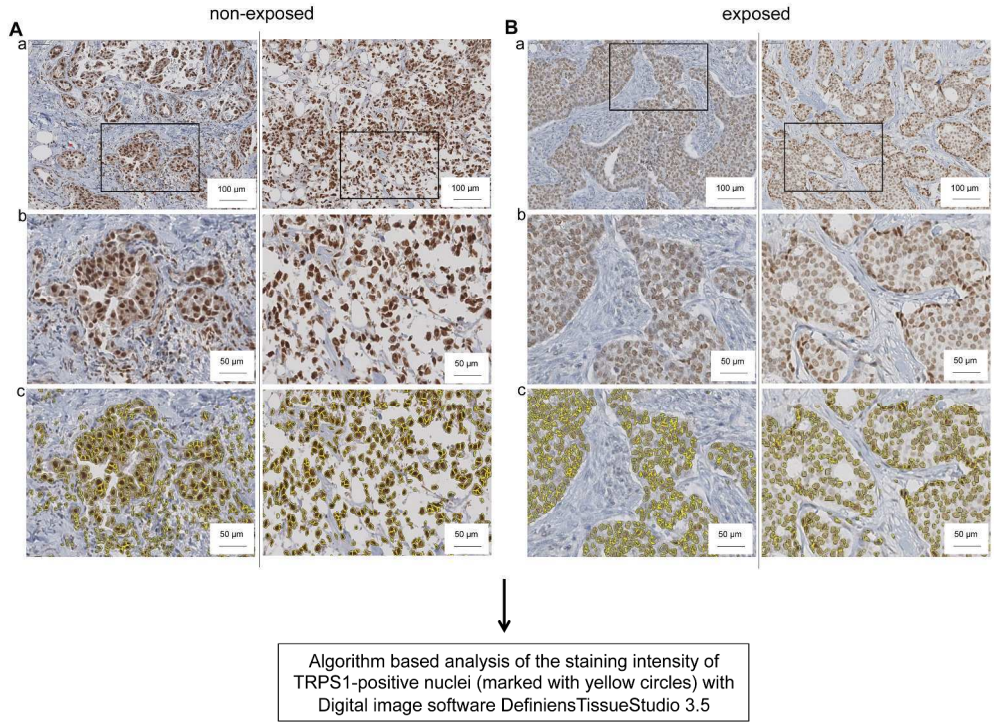

Fig. 2) Digital image analysis of immunhistochemically stained FFPE tumour sections from non-exposed and exposed breast cancer samples using an antibody against TRPS1. A/B: Two representative immunohistochemically stained breast carcinoma cases are shown for non-exposed (A) and exposed (B) cases. Image details of Aa and Ba (black frames) are shown in Ab and Bb. Detection and quantification of TRPS1 stained nuclei was performed using the digital image software Definiens. Nuclei of tumour cells, for which the staining intensities were calculated based on the algorithm, are labeled in yellow (Ac; Bc).

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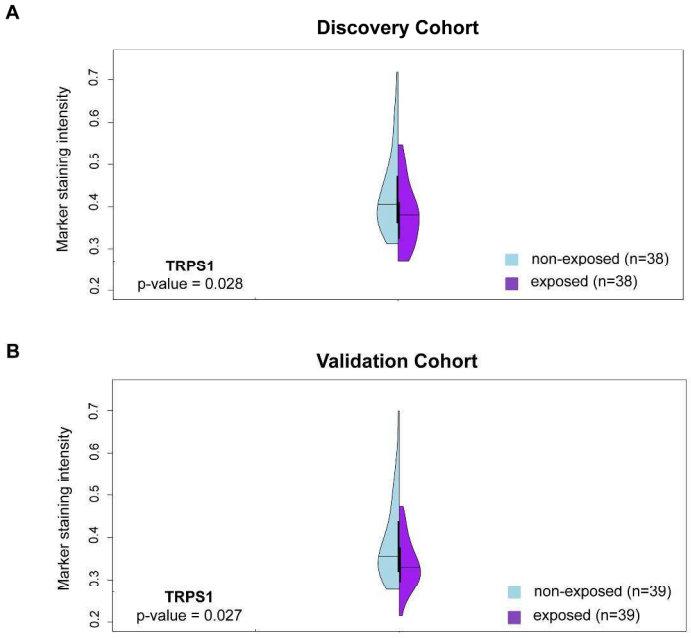

Fig. 3) Significantly increased TRPS1 protein expression represented by the marker staining intensity was observed in breast cancer tissues from the non-exposed groups (light blue) compared to the exposed groups (purple) in the discovery (A, p-value=0.028) and validation cohorts (B, p-value=0.027). p-values were calculated using the partial differential test considering inter tumour heterogeneity.

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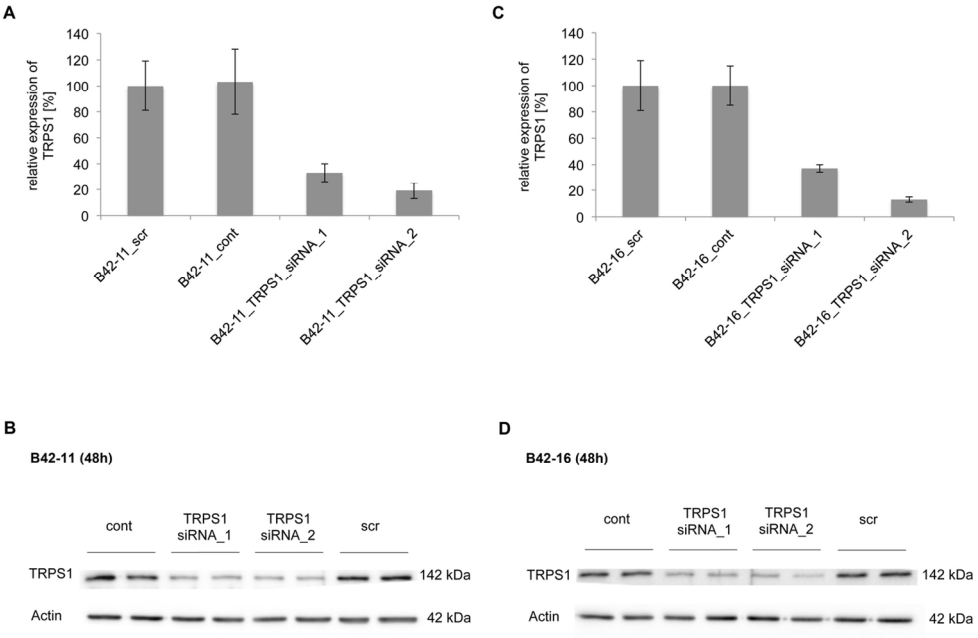

Fig. 4) A/C: Levels of TRPS1-mRNA-expression in untransfected (cont), scrambled- siRNA transfected (scr) and TRPS1-siRNA transfected B42-11 and B42-16 cells 48h after transfection. B/D: Western Blot images show levels of TRPS1-protein-expression in untransfected (cont), scrambled-siRNA transfected (scr) and TRPS1-siRNA transfected B42-11 and B42-16 cells 48h after transfection.

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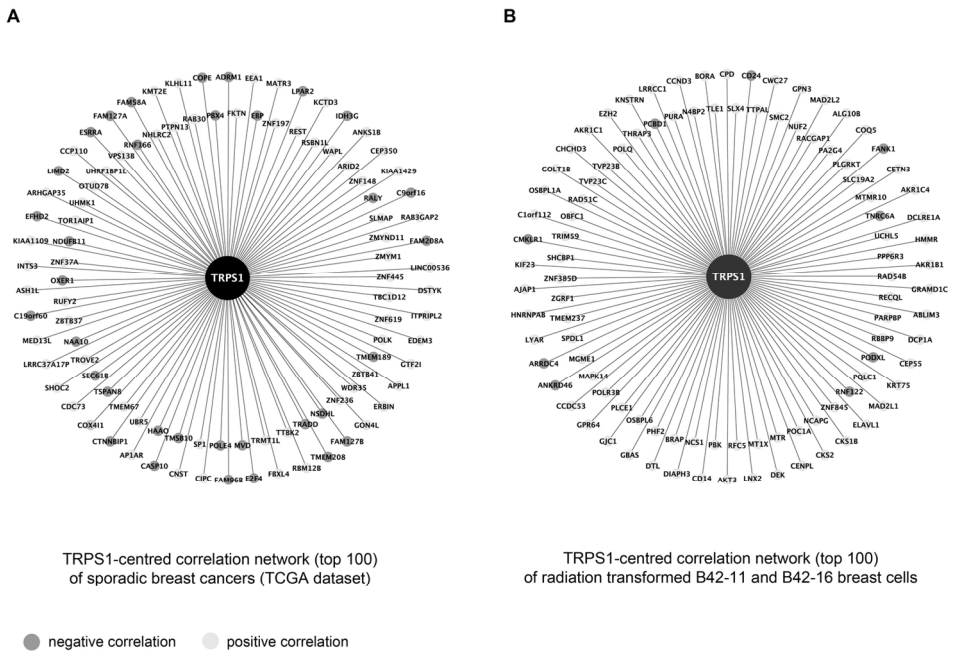

Fig. 5) TRPS1-centred correlation networks consisting of the top 100 correlating genes with a FDR < 0.05; The expression of genes labelled with dark grey circles showed negative correlation with TRPS1-expression and that of genes labelled with light grey circles showed positive correlation with TRPS1-expression. A: TRPS1-centred correlation network based on global mRNA expression data from matched sporadic breast cancers of the publicly available TCGA dataset. B: TRPS1-centred correlation network based on microarray gene expression data from B42-11 and B42-16 untransfected, scrambled-siRNA transfected and TRPS1-downregulated cells.

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