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Higher risk for carcinogenesis for residents populating the isotope-contaminated territories as assessed by Nano String Gene Expression Profiling

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Abstract

Risk for carcinogenesis in several generations of residents from areas contaminated with radioactive materials was assessed via gene expression profiling of the p53 gene network by the NanoString technology (United States). In total, 24 genes of the *TP53* network showed significant differences between the observation and reference groups of subjects. The most significant differences were observed for five genes: *ST13, IER3, BRCA1, LRDD,* and *MRAS.* The study confirmed again that the NanoString technology provides overt advantages in terms of sensitivity and throughput when extended gene networks are examined.

Introduction

The NanoString technology has been developed and patented by Krassen Dimitrov and Dwayne Dunaway [1]. The gist is using fluorescent molecular barcodes to detect gene transcripts via fluorescence microscopy, which is capable of visualizing up to 800 nucleic acids per sample. An advantage of the technology is that enzymatic reactions and dilutions are avoided in measuring the mRNA amounts, and measurements are consequently far more sensitive and rapid. Dimitrov and Dunaway's experiments have shown that the results are highly reproducible in two replicate assays, yielding a replication correlation coefficient of 0.999, a detection limit between 0.1 and 0.5 fM, and a linear dynamic range of 500-fold. Comparisons have demonstrated that the NanoString technology is more sensitive than DNA microarray-based assays and is far simpler and have a higher throughput than real-time PCR using TaqMan and SYBR Green probes.

The NanoString technology is therefore highly efficient in studying large gene networks. Such studies are important to perform in various polygenic disorders, in particular, those due to exposure to external mutagenic factors. Accordingly, the studies may play a material role in evaluating genetic defects and risk for carcinogenesis in people chronically exposed to radiation. The cancer-related p53 gene network is of particular interest to study.

Mammalian p53 originates from a transcription factor that occurred in ancient multicellular organisms and regulated transcription in response to stress as its primary function. A tumor suppressor role of p53 was probably acquired relatively recently in evolution, as an adaptation of organisms that have prolonged lifespans and accumulate many somatic mutations [2]. The significant role that p53 plays in maintaining genome stability is clearly evident from that fact that mutant p53 is found in approximately half of human tumors. More than 10 million tumor samples have been observed to carry inactivated p53, and about the same number of tumors contained other inactivated proteins [3]. However, tumor suppression is not the only function of p53. The protein performs many functions, regulating cell migration, autophagy, anaerobic and aerobic glycolysis, and other cell metabolic pathways [4-6]. In addition, p53 modulates cell differentiation [7]. A p53 deletion suppresses terminal differentiation of skeletal muscles, many hematopoietic cell lineages, renal epithelial cells, thyrocytes, and oligodendrocytes, as well as axon growth and regeneration [8,9]. While often playing a central crucial role in carcinogenesis, the p53 gene (*TP53*), as any gene, it is involved in interacting with many other genes and their protein products and, with each of them, forms positive and negative feedbacks and compensatory mechanisms, which coordinate the function of the total *TP53* network. It is nearly meaningless to study one gene alone without its network, be it even the most important gene.

Objective

To demonstrate that gene expression profiling of the p53 gene network with the Nano String technology (United States) is suitable

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for evaluating risk for carcinogenesis in various generations of residents from areas contaminated with radioactive material.

Materials and methods

To assess higher risk for carcinogenesis, we examined 36 residents from Russian areas contaminated with radioactive material as a result of the Chernobyl meltdown. This observation group included 13 mothers, who were born from 1969 to 1987 and formed generation I, and their children (23 in total, one to three per mother), who were younger than 18 years of age and formed generation II. The criteria for inclusion in the monitoring group were also: the permanent residence of patients in the radionuclide contamination region with a soil contamination level of cesium-137 from 556 kBq/m^2 to 1665 kBq/m^2 (including for the I generation presence at the time of the accident in the indicated Region). Patient generations I and II did not have radiation-induced diseases, including oncopathology.

A reference group included 12 subjects (6 of generation I and 6 of generation II, similar in age) from areas free from radioactive pollution.

To profile expression of the p53 gene network, RNA was isolated from lymphocytes of fresh peripheral blood samples of the 48 subjects. RNA was extracted from peripheral blood with PureLink® Total RNA Blood Kit. Concentration and quality of samples were detected by Implen NanoPhotometer Pearl and gel-electrophoresis.

Samples were run on the nCounter Analysis System (NanoString Technologies), according to the manufacturer's protocol in a total of 4 runs. Each sample contained at least 100 ng of RNA. All hybridization reactions were 24-26 hours long; all PrepStation run were held on high sensitivity mode and all counts were gathered by scanning on MAX mode for 555 fields of view per sample.

No nCounter samples were flagged by nSolverAnalysisSoftware (NanoString Technologies) for quality control. nCounter data was normalized with inner nCounter-provided positive controls on the basis of RNA yields in each sample. No normalization factors were outside the NanoString-recommended range of 0.3-3. Thresholds to identify expressed genes from background noise were then calculated as the median of the maxima of the negative control probe measurements for each cell type.

We did not confirm our data from nCounter with different methods. Up to date there are numerous papers, that show absolute advantage of Nanostring technology over PCR or microarrays [10,11].

The resulting gene expression data were normalized and statistically analyzed. The analysis was performed using the NCSS 11 statistical package. The results for each gene and sample were checked for normal distribution using the Shapiro-Wilk test. If the distribution with significance level p <0.05 was considered normal, we used the Student's t-test for comparison. If there were not enough evidence for considering the distribution to be normal, we used the nonparametric Mann-Whitney test for comparison.

The p53 gene network includes a huge number of genes, and their list is continuously increasing. To design the panel for the network, we selected the 102 genes most tightly associated with *TP53* at the molecular and functional levels according to published data. Six housekeeping genes were chosen as controls. The genes are summarized in the Extended Data Table 1.

Expression profiling of the p53 gene network in generations I and II of residents from radioactive areas was carried out with a digital nCounter Analysis System (NanoString Technologies, United States).

The resulting gene expression data were normalized and statistically analyzed. The analysis was performed using the NCSS 11 statistical package. Data of the two groups were compared by the Mann–Whitney nonparametric test.

Results

The *TP53* expression level did not significantly differ between the observation and reference groups. However, expression profiling revealed 24 genes that showed statistically significant differences between the two groups (Figure 1).

The most significant differences were observed for five genes: *ST13, IER3, BRCA1, LRDD,* and *MRAS* (Figure 2).

Stratified by generation, the expression levels of all five genes significantly differed significantly between the two groups in both generation I and generation II (Figures 3 and 4).

The two generations did not significantly differ in gene expression levels (Figure 5).

Discussion

Thus, our results show that *TP53* expression in the two generations of residents from radioactive areas does not differ from

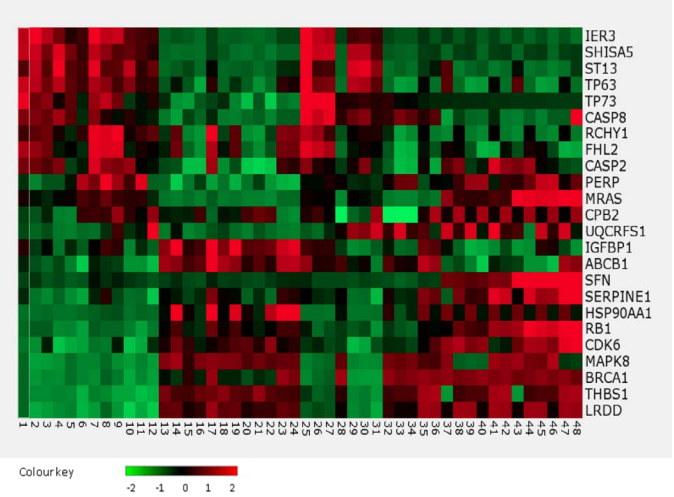

Figure 1. The heatmap of the p53 gene network that showed significant ($p< 0.05$) upregulation or downregulation

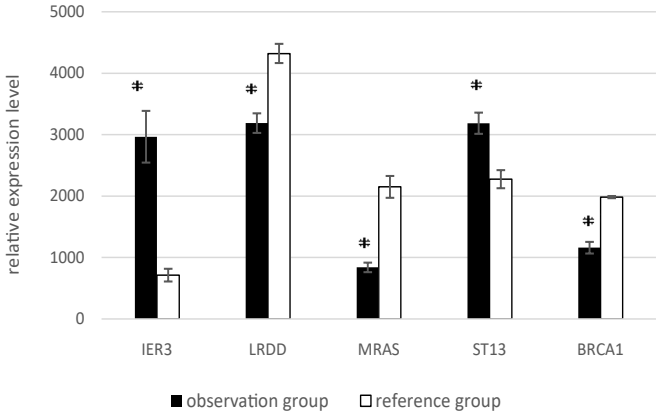

Figure 2. Mean expression levels of *ST13, IER3, BRCA1, LRDD, MRAS* in the observation and reference groups. Hereafter, the whiskers show the standard error of the mean (SEM)); * - for significant ($p< 0.05$) differences between groups.

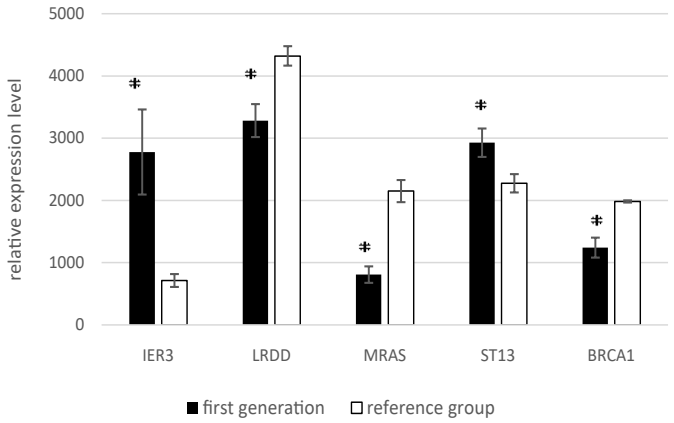

Figure 3. Mean expression levels of the five genes in generation I to the reference group.

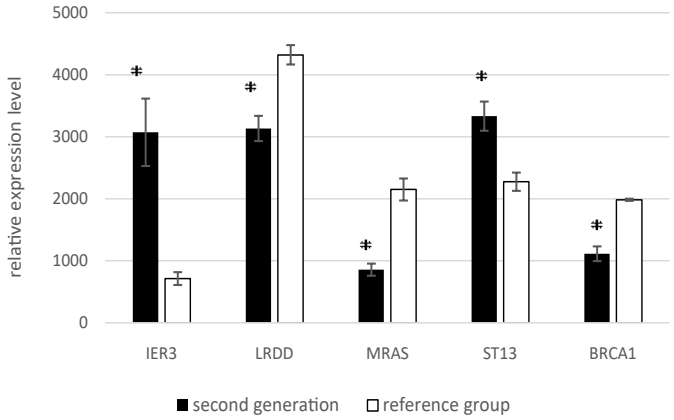

Figure 4. Mean expression levels of the five genes in generation II to the reference group.

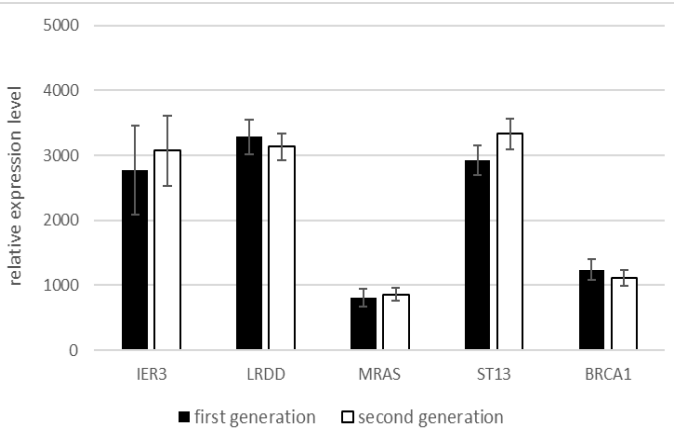

Figure 5. Mean expression levels of the five genes in generations I and II of the observation group.

that in the reference group. However, the analysis of the p53 gene network demonstrated that significant changes (upregulation or downregulation) occur in expression of several functionally important genes and are possibly transmitted from one generation to the next one.

The analysis using the NanoString technology revealed 24 genes with significant changes in expression in the p53 gene network. Five genes showed the most substantial changes.

A significant increase in the level of gene expression:

IER3 is expressed to a high level in many tumor tissues. Higher levels of its protein product may increase risk for tumorigenesis. Its role in regulating apoptosis is a matter of active discussion. *IER3* expression is tightly associated with mutations of *TP53, Ki-67,* and *EGFR,* leading to a greater tumor size and a more advanced tumor stage [12].

ST13 which inhibits tumor growth in various cancer; *ST13* downregulation may increase risk for gastric and colorectal carcinomas [13]. The gene codes for an adaptor protein that interacts with the heat shock proteins and is involved in glucocorticoid receptor assembly via many molecular chaperones. The expression of this gene in the observation group is increased relative to the values in the reference group, which may be the result of the response of the organism to the long-existing high risk of developing a tumor or radiation-induced activation of carcinogenesis processes.

The expression level of the following genes is significantly lowered:

BRCA1 downregulation may increase risk for breast and ovarian cancers. The gene codes for a phosphoprotein that maintains genome stability and acts as a tumor suppressor. *BRCA1* mutations are found in approximately 40% of inherited breast cancer cases. Downregulation or lack of *BRCA1* expression is observed in the majority of breast cancer cases [14].

LRDD Downregulation of the gene may decrease apoptotic activity in response to genotoxic agents. The protein product is also known as a p53-induced protein with a death domain. The protein can interact with other death domain-possessing proteins (such as FADD or MADD) in signaling cascades and leads to spontaneous activation of caspase 2 [15,16].

MRAS Downregulation of the gene also may decrease apoptotic activity. The gene codes for a protein of the Ras family (small GTPases). The product is a membrane protein and acts as a transducer in many processes, including cell growth and differentiation. Ras deregulation is associated with many cancers. The protein plays an important role in the TNF-α and MAP kinase signaling pathways [provided by RefSeq, Nov 2011]

Thus, our results show that *TP53* expression in the two generations of residents from radioactive areas does not differ from that in the reference group. However, the analysis of the p53 gene network demonstrated that significant changes (upregulation or downregulation) occur in expression of several functionally important genes and are possibly transmitted from one generation to the next one.

Changes in expression of four out of the five genes in question relative to the reference group suggest higher risk of carcinogenesis for residents of areas contaminated with radioactive materials. The other genes need further investigation in larger samples of subjects and at the DNA level with the use of high-throughput sequencing.

A correlation analysis of the results is now in progress; its results will soon be published.

Our pilot study with the NanoString technology makes it possible to assume a transgenerational transmission for specific consequences of radiation exposure and, therefore, higher risk of carcinogenesis.

Our study testifies again that the NanoString technology in advantageous in terms of both sensitivity and throughput for examining large gene networks.

Extended Data Table 1. Genes included in the p53 panel

Gene	Gene name	Transcript ID
CLTC	clathrin heavy chain	NM_001101.2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_001916.3
GUSB	glucuronidase beta	NM_002046.3
HPRT1	hypoxanthine phosphoribosyltransferase 1	NM_000194.1
PGK1	phosphoglycerate kinase 1	NM_000291.2
TUBB	tubulin beta class I	NM_006086.2
ABCB1	ATP binding cassette subfamily B member 1	NM_001111.3
ACTA2	actin, alpha 2, smooth muscle, aorta	NM_001033049.1
AKT1S1	AKT1 substrate 1	NM_018702.3
APAF1	apoptotic peptidase activating factor 1	NR_110620.1
ATF3	activating transcription factor 3	NM_181869.1
ATM	ATM serine/threonine kinase	NM_001644.3
ATR	ATR serine/threonine kinase	NM_004707.2
ADGRB1	adhesion G protein-coupled receptor B1	NM_138292.3
BAX	BCL2 associated X	NM_004993.5
BBC3	BCL2 binding component 3	NM_004322.3
BCL2	BCL2, apoptosis regulator	NM_001184900.1
BCL6	B-cell CLL/lymphoma 6	NM_199141.1
BID	BH3 interacting domain death agonist	NM_032977.3
BRCA1	BRCA1, DNA repair associated	NM_032992.2
BTG2	BTG anti-proliferation factor 2	NM_001228.4
CASP2	caspase 2	NM_012115.2
CASP3	caspase 3	NM_001229.2
CASP8	caspase 8	NM_012117.1
CASP9	caspase 9	NM_018237.3
CCND1	cyclin D1	NM_001259.6
CCNE1	cyclin E1	NM_001127183.1
CDK1	cyclin dependent kinase 1	NM_000492.3
CDK2	cyclin dependent kinase 2	NM_001908.3
CDK4	cyclin dependent kinase 4	NM_018947.4
CDK6	cyclin dependent kinase 6	NM_001349.2
CDKN1A	cyclin dependent kinase inhibitor 1A	NM_014314.3
CDKN2A	cyclin dependent kinase inhibitor 2A	NM_001304794.1
CHEK1	checkpoint kinase 1	NM_080876.3
CHEK2	checkpoint kinase 2	NM_013302.3
COL18A1	collagen type XVIII alpha 1 chain	NM_004094.4
CPB2	carboxypeptidase B2	NM_001568.2
CRY1	cryptochrome circadian clock 1	NM_001214903.1
CSNK1A1	casein kinase 1 alpha 1	NM_003824.2
CSNK1D	casein kinase 1 delta	NM_198549.1
DDB2	damage specific DNA binding protein 2	NM_004960.2
E2F1	E2F transcription factor 1	NM_001161706.1
E2F3	E2F transcription factor 3	NM_001516.4
FAS	Fas cell surface death receptor	NM_020771.2
FHL2	four and a half LIM domains 2	NM_006044.2
GADD45A	growth arrest and DNA damage inducible alpha	NM_016299.2
GAK	cyclin G associated kinase	NM_001548.3
GAL3ST3	galactose-3-O-sulfotransferase 3	NM_001031683.2
GDF15	growth differentiation factor 15	NM_000629.2
GTSE1	growth differentiation factor 15	NM_000874.3
HDAC1	histone deacetylase 1	NM_016123.1
HIC1	HIC ZBTB transcriptional repressor 1	NM_001145805.1
HIF1A	hypoxia inducible factor 1 alpha subunit	NM_139068.2
HRAS	HRas proto-oncogene, GTPase	NM_020746.3
HSPA4L	heat shock protein family A (Hsp70) member 4 like	NM_006617.1
IER3	immediate early response 3	NM_014932.2
IGFBP1	insulin like growth factor binding protein 1	NM_007363.4
IGFBP3	insulin like growth factor binding protein 3	NM_001105250.1
IGFBP4	insulin like growth factor binding protein 4	NM_020401.2
ITSN1	intersectin 1	NM_001032409.1

KAT2B	lysine acetyltransferase 2B	NM_001008211.1
LRDD	p53-induced death domain protein 1	NM_013232.2
MAPK8	mitogen-activated protein kinase 8	NM_138575.3
MDM2	MDM2 proto-oncogene	NM_002675.3
MRAS	muscle RAS oncogene homolog	NM_014330.2
NFKBIB	NFKB inhibitor beta	NM_032833.3
NQO1	NAD(P)H quinone dehydrogenase 1	NM_002719.3
PCNA	proliferating cell nuclear antigen	NM_004157.2
PERP	PERP, TP53 apoptosis effector	NM_002794.3
PIGS	phosphatidylinositol glycan anchor biosynthesis class S	NM_174871.2
PLK3	polo like kinase 3	NM_014455.3
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	NM_005785.2
PPM1D	protein phosphatase, Mg2+/Mn2+ dependent 1D	NM_080657.4
PRKAB1	protein kinase AMP-activated non-catalytic subunit beta 1	NM_012426.3
PTEN	phosphatase and tensin homolog	NM_005066.2
PTK2	protein tyrosine kinase 2	NM_006938.2
RB1	RB transcriptional corepressor 1	NM_007315.2
RCHY1	ring finger and CHY zinc finger domain containing 1	NM_007375.3
RDBP	negative elongation factor complex member E	NM_001146213.1
RFWD2	ring finger and WD repeat domain 2	NM_148910.2
RPRM	reprimo, TP53 dependent G2 arrest mediator homolog	NM_003264.3
RRM2B	ribonucleotide reductase regulatory TP53 inducible subunit M2B	NM_003265.2
SERPINB2	serpin family B member 2	NM_003844.2
SERPINB5	serpin family B member 5	NM_001204344.1
SERPINE1	serpin family E member 1	NM_012470.2
SESN1	sestrin 1	NM_001128917.1
SESN2	sestrin 2	NM_000546.2
SESN3	sestrin 3	NM_139075.3
SFN	stratifin	NM_145803.1
SHISA5	shisa family member 5	NM_001007278.1
SIAH1	siah E3 ubiquitin protein ligase 1	NM_017672.2
SIAH2	siah E3 ubiquitin protein ligase 2	NM_018955.2
SIAH3	siah E3 ubiquitin protein ligase family member 3	NM_001035247.2
SIRT1	sirtuin 1	NM_152586.3
ST13	ST13, Hsp70 interacting protein	NM_001204401.1
STEAP3	STEAP3 metalloreductase	NM_015269.2
THBS1	thrombospondin 1	NM_014413.2
TIMP3	TIMP metallopeptidase inhibitor 3	NM_002759.1
TNFRSF10B	TNF receptor superfamily member 10b	NM_004836.3
TP53	tumor protein p53	NM_001013703.2
TP63	tumor protein p63	NM_001198801.1
TP73	tumor protein p73	NM_002140.3
TSC2	tuberous sclerosis 2	NM_000548.3:95
UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	NM_006003.2:854
VCAN	versican	NM_004385.3:9915
HSPA1A	heat shock protein family A (Hsp70) member 1A	NR_028272.1
HSP90AA1	heat shock protein 90 alpha family class A member 1	NR_131012.1
ZMAT3	zinc finger matrin-type 3	NM_022470.2

Thus, an important and pressing task now is to continue the study with more gene networks involved and a more detailed analysis of their genes.

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