Association Between Resistance to Mammary Cancer Development and Upregulation of DNA Damage Response Genes

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Abstract: Rat strains differ strikingly in susceptibility to cancer. The rat strain WKY is highly resistant, while the SPRD-Cu3 strain is susceptible to chemically-induced mammary cancer. We previously showed that two chromosome regions (from chromosomes 5 and 18) contain quantitative trait loci (QTLs) controlling mammary cancer susceptibility. Here we tested the hypothesis that mammary cancer resistance is associated with a prompt and efficient DNA damage response (DDR) leading to a robust anti-cancer barrier. We also hypothesized that an efficient response to carcinogenic [(7,12-dimethyl benz[a]anthracene (DMBA)] treatment could be accompanied by gene activation and changes in mRNA levels. We thus compared the mRNA levels of several genes involved in the DDR in the mammary tissue of DMBA-treated and control WKY, SPRD-CU3 and congenic female rats. Our observations show that DMBA-treatment induces a dramatic increase in the level of several DDR mammary tissue mRNAs in rat strains that are resistant to mammary cancer, but not in the susceptible SPRD-Cu3 strain. Some of the upregulated genes are tumour supressor genes, such as *Tp53* and *Brca1*. Several genes involved in the DDR are thus subject to regulations impacting their mRNA level and our results strongly support the hypothesis that the DDR is a barrier in early tumorigenesis.

Keywords: Mammary, cancer, rat, DNA damage.

INTRODUCTION

Breast cancer affects about 10% of women in industrialized countries such as the USA, Australia, and several Western Europe countries [1-4]. The etiology of this disease and its initiation mechanisms remain elusive, but it is quite clear that breast cancer is a complex trait, probably resulting from the interplay between genes and environmental factors, including diet and life habits [5-9]. Linkage and association studies have identified over 25 genes that modulate breast cancer risk [for reviews, see 7,10]. Despite these remarkable discoveries, most of the familial risk remains unexplained [7,11,12].

Mouse and rat provide us with inbred strains that exhibit a wide range of susceptibility to mammary cancer and are useful models for studying mammary cancer susceptibility [10,13]. Indeed, susceptibility (or resistance) loci can be mapped by different means, the causative genes can then be identified and the role of their human orthologues can be evaluated in human populations [14,15]. In addition, the biological mechanisms underlying the development of mammary tumours can be experimentally analysed, thereby generating information potentially useful on the clinical level. Several quantitative trait loci (QTLs) controlling susceptibility induced mammary cancer have been identified in the rat [for recent reviews, see 10,13]. We previously reported the identification of several loci controlling mammary cancer susceptibility, and more precisely controlling multiplicity of 7,12-dimethyl benz[a]anthracene (DMBA)-induced mammary tumours: QTLs were notably assigned to rat chromosomes 5 and 18 (*Mcstm1* and *Mcstm2*) [16,17]. DMBA is an indirect carcinogen, which has to be metabolised to generate mutations and tumorigenesis, notably through the formation of bulky DNA adducts [18]. These DNA damages can be repaired by the nucleotide excision repair pathway (NER), a major defense system able to eliminate several types of DNA lesions [19,20]. Rat mammary cancer susceptibility is a tissue specific and cell autonomous phenotype [21] and this phenotype could not be related to differences in DMBA metabolism or DNA binding [22]. Furthermore, susceptibility to chemically- or physically-induced mammary cancer is not dependent on the nature or the identity of the inducing carcinogen agent and resistance to mammary cancer (in the COP and WKY strains for instance) is independent of the inducing agent [reviewed in 10]. These observations strongly suggest that the genes underlying differences in mammary cancer susceptibility are not associated with one specific type of DNA lesion repair but, instead, are acting in more global pathways of the DNA damage response (DDR) and of genome integrity maintenance, including surveillance mechanisms such as cell-cycle blockade, apoptosis or immunity, which are very likely protective mechanisms against cancer [23-26]. In humans, several breast cancer susceptibility genes are involved in the DNA damage network [27] and early tumour lesions express signs of a DDR, such as the phosphorylated proteins ATM, CHK2 and TP53 [28,29]. These observations indicate that this hypothesis is not restricted to animal models and the DDR has been proposed to provide a barrier against progression to cancer [28-30]. Interestingly, and along similar lines, a recent study showed that human breast cancer genomes are characterized by diverse rearrangements, most of which are intrachromosomal ones with breakpoints located within 2 megabases of each other, suggesting the presence of a defect in DNA maintenance [31].

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The COP and WKY strains seem to differ in the mechanisms ensuring mammary cancer resistance. Indeed, upon chemical carcinogen treatment, COP females exhibit mammary preneoplastic lesions which disappear after several weeks [32], a phenomenon which could be explained by a form of immune surveillance, while in the same circumstances, WKY females do not show any lesion [16]. These observations led us to hypothesize that in the WKY strain, DNA injury induces a prompt and efficient DDR leading to a robust anti-cancer barrier. We also hypothesized that an efficient response would be accompanied by gene activation and changes in mRNA levels. We thus compared the mRNA levels of NER, cell-cycle checkpoints, cell growth arrests or apoptosis genes, in the mammary tissue of DMBA-treated and control females from several rat strains, focusing on WKY (resistant), SPRD-Cu3 (susceptible) and two related congenic strains exhibiting a substantial reduction in DMBA-induced mammary tumour numbers. Each of these congenic strains carries, on the SPRD-Cu3 genetic background, a WKY-derived chromosome segment (from chromosome 5 or from chromosome 18). These two congenic strains [SPRD-Cu3-Mcstm1 (in brief, C5.Mcstm1) and SPRD-Cu3-Mcstm2 (in brief, C18.Mcstm2)] exhibit a reduction of 65% and 33% in DMBA-induced tumour multipliciyty, respectively [16,17].

MATERIAL AND METHODOLOGY

Animals

The SPRD-Cu3 (a curly mutant of Sprague-Dawley rats) and WKY/E56 (in brief: WKY) rats were obtained from the Medizinische Hochschule Hannover, Institut fur Versuchstierkunde und Zentrales Tierlaboratorium (Hannover, Germany). The congenic strains were described previously [17,33]: the strains were derived from SPRD-Cu3, the SPRD-Cu3.WKY-Mcstm1 (C5.Mcstm1) strain containing a WKY chromosome 5 segment, from D5Rat124 to *Pla2g2a*, while the SPRD-Cu3.WKY-Mcstm2 (C18.Mcstm2) strain contains a WKY chromosome 18 segment, from D18Wox8 to D18Rat44.

Induction of Mammary Cancer, RNA Extraction, Reverse Transcription and Real-Time PCR

All methods used were described previously [33]; the RNA concentration and quality were assessed using a RNA Lap Chip (Bioanalyser Agilent 2100 Expert, Agilent Technologies, Diegem, Belgium) and reverse transcription and quantitative PCR were carried out using the Superscript™ III Platinum® two step qRT-PCR Kit (Cat. No. 11734-050 from Invitrogen, Merelbeke, Belgium) according to the manufacturer instructions. Gene specific TaqMan® probes (Gene Expression Assays) were purchased from Applied Biosystems (Foster City, USA). The probes used were: Rn01498470_m1 (*Ercc1*), RN00514776_m1 (*Ercc2*), Rn01439904_m1 (*Xpa*), Rn01425435 (*Xpc*), Rn01421965_m1 (*Atm*), Rn01223644_m1 (*Atr*), Rn01644545 (*Brca1*), Rn00589669 (*Chk1*), Rn01516538 (*Cslpn*), Rn01448354_m1 (*Mre11a*), Rn00594037 (*Nbn*), Rn00755717_m1(*Tp53*), Rn99999089 (*Cdkn1a/p21)*, Rn006676469_m1 (*Cdkn2a/p16*), Rn01433231 (*Cdkn2a/p19*), Rn99999121_m1 (*Gadd45a*), Rn00597992_m1 (*Bbc3/Puma)*, Rn01494552_m1 (*Pmaip1/ Noxa*). At least three distinct animals from each strain were used and assays were made in triplicates. All results were normalized to beta2-microglobulin expression (Rn00560865 _m1). Data were analysed using the Relative Quantification Software of the Applied Biosystems 7300 equipment and the SigmaStat 3.0 statistical analysis software.

RESULTS

Eighteen genes were selected for this analysis: they are listed in Table **1**. Taking into account the availability of relevant probes for RT-qPCR, the rationale was to test key genes involved in NER, as well as genes acting downstream in the DDR and having known or assumed connections to NER. Four NER genes were selected, including *Xpc*, because the XPC protein initiates NER and has also been shown to be required for the association of the ATM protein to damaged DNA [34] (ATM, and the related protein ATR play important roles in cell cycle checkpoint regulation and the human *ATM* gene is a breast cancer susceptibility gene). *Xpd* was also selected, because the XPC protein binds TP53, a well-known tumour suppressor protein [35,36] the absence of which predisposes to human breast cancer [8]. The p53 protein also plays a role in NER [37,38]. In addition to the *Tp53* gene, seven other genes involved in cell cycle checkpoints were chosen, including *Brca1*. Four transcripts controlling cell cycle progression (*p21*, *p16* and *p19Arf, and Gadd45a*), three of which are tumour suppressors [39,40] as well as two apoptosis-specific genes [*Bbc3 (Puma)* and *Pmaip1* (*Noxa*) [41,42]] were also selected.

Preliminary tests showed that important and significant increases in mRNA levels of several transcripts were detected in WKY mammary tissue 10 days after DMBA treatment (i.e. in 65-day old females), with, in several instances, returned to control values in later times. This time point was thus chosen for all comparisons.

Strikingly, three of the four NER transcripts (all but *Xpa*) showed increased levels (4 to 6-fold) in 65-day-old DMBA-treated WKY females, relative to aged-matched untreated animals (Fig. **1A**). Remarkably, no DMBA-induced increase was detected in the level of any of these transcripts in the susceptible strain, SPRD-Cu3, while the two congenic strains showed several-fold increases in both *Ercc1* and *Ercc2* levels. The strain C18.Mcstm2 also showed a 4-fold increase of the *Xpc* transcript level.

Among the eight cell cycle checkpoint mRNA's, four were stimulated in DMBA-treated WKY females, namely: *Atm* (x1.5), *Brca1* (x10), *Chk1* (x4) and *Tp53* (x2.5) (Fig. **1B**). In the SPRD-Cu3 strain, no stimulatory effect of DMBA was observed (on the contrary, the *Tp53* transcript level was lower after DMBA treatment). With respect to the two congenic strains, the *Atm* and *Tp53* mRNA levels increased in C18.Mcstm2 but not in C5.Mcstm1 (just as *Xpc*), while the levels of the *Brca1* and *Chk1* transcripts were enhanced in the two congenic strains by DMBA-treatment.

The *Cdln1a* (*p21*) level was stimulated by DMBA treatment in the WKY and congenic strains (from 2-fold in C18.Mcstm2) to 8-fold in WKY) (Fig. **1C**). On the other

Table 1. Genes Analyzed

Gene Symbol (alias)	Name	Chromosome Position (rat)
NER		
Ercc1	Excision repair cross-complementing rodent repair deficiency, complementation group 1	1q21
Ercc2 (*Xpd*)	Excision repair cross-complementing rodent repair deficiency, complementation group 2	1q21
Xpa	Xeroderma pigmentosum, complementation group A	5q22
Xpc	Xeroderma pigmentosum, complementation group C	4q34
CHECKPOINTS		
Atm	Ataxia telangiectasia mutated homologue (human)	8q24
Atr	Ataxia telangiectasia and *Rad3* related	8q31
Brca1	Breast cancer 1	10q32
Chk1	Checkpoint kinase 1 homologue	8q21
Clspn	Claspin homologue	5q36
Mre11a	MRE11 meiotic recombination 11 homologue A	8q11
Nbn (Nbs1)	Nibrin, Nijmegen breakage syndrome 1	5q13
Tp53	Tumor protein p53	10q24
CELL CYCLE ARREST		
Cdkn1a (p21)	Cyclin-dependent kinase inhibitor 1A, Cip1, Waf1)	20p12
Cdkn2a (p16)	Cyclin-dependent kinase inhibitor 2A, Ink4a	5q32
Cdkn2a (p19)	Cyclin-dependent kinase inhibitor 2A, p19Arf	5q32
Gadd45a (Ddit1)	Growth arrest and DNA-damage-inducible, alpha	4q31-q33
APOPTOSIS		
Bbc3 (Puma)	Bcl-2 binding component 3	1q21
Pmaip1 (*Noxa*)	Phorbol-12-myristate-13-acetate-induced protein 1	18q12

hand, the *Gadd5a* mRNA level gene was stimulated (x4) in WKY females, while it was reduced in SPRD-Cu3 females (x0.5) and was unchanged in the two congenic strains. The levels of the *p16* and *p19* transcripts were very low and no significant change could be detected.

Finally, the two apoptosis genes showed clear-cut stimulation upon DMBA treatment of WKY or congenic females (up to x6) (except *Pmaip1* in C5), with no increase in SPRD-Cu3 females (Fig. **1D**). This DMBA-induced stimulation was particularly striking in the case of *Bbc3* (*Puma*).

DISCUSSION

This work demonstrates that DMBA-treatment induces a dramatic increase in the level of several mammary tissue mRNAs involved in the DDR, at least in rat strains that are resistant to mammary cancer. Taking into account the heterogeneity of the mammary tissue, one explanation could be that the relative contributions of different cell sub-population is modified as a consequence of carcinogen injury, the rise in the mRNA levels reflecting some enrichment in cells over-expressing the DMBA-treatment upregulated genes. This explanation is not very attractive. Indeed, it seems unlikely that any cell sub-population would preferentially over-express this precise set of genes. Therefore, a more reasonable interpretation is that, as a response to DNA damage, mammary cells increase their content in several mRNAs encoding proteins engaged in DDR, by stimulating gene transcription and/or by stabilizing the mRNAs. DDR is generally viewed as a process involving cascades of critical and complex post-translational modifications, as well as TP53-dependent transcriptional activation of genes such as *Cdkn1*, *Gadd45a, Xpc*, *Pmaip1* (*Noxa*) and *Bbc3 (Puma)* [38,41-46]. The *Tp53* gene itself is subject to complex transcriptional and post-transcriptional controls [46] and our work thus brings a new example of upregulation of the *Tp53* gene itself and of several TP53 target genes. However, several of the genes we found to be upregulated by DMBA treatment are not known TP53 targets (*Ercc1*, *Ercc2*) [47] or their products act upstream TP53 (*Atm*, *Brca1* and *Chk1*) [45]. Upregulation of these genes thus cannot be explained by TP53 activation and to our knowledge, upregulation of these genes in carcinogen-treated cells has not been reported previously. This observation deserves further investigations, all the more so since several of these genes are mammary cancer susceptibility genes, such as *Brca1*. *Brca1* encodes a transcriptional coactivator of genes involved in DNA repair and cell cycle arrest, including

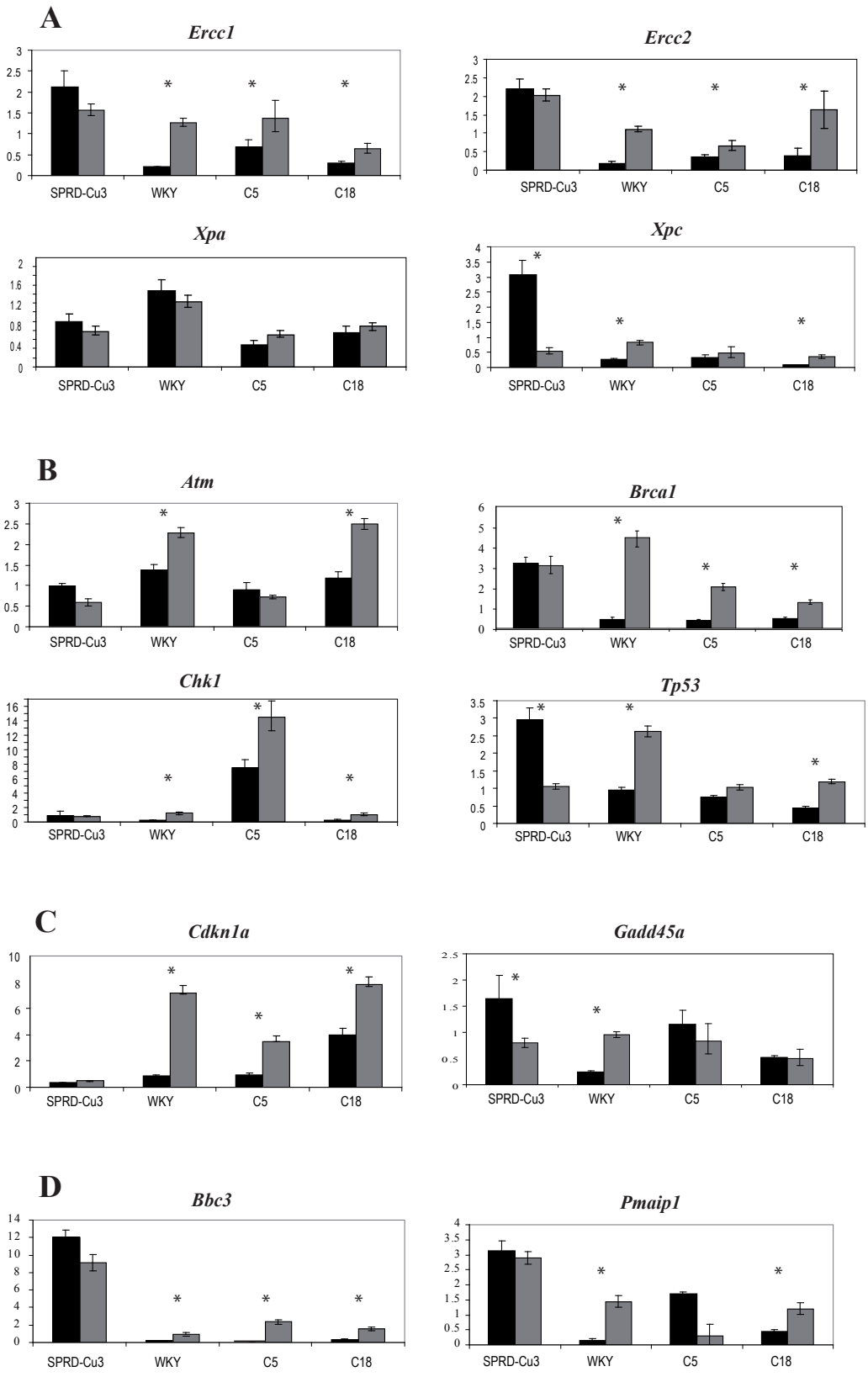

Fig. (1). Relative level (±SD) of mRNAs in 65-day old mammary glands of control (black rectangles) and of DMBA-treated (grey rectangles) rats in the parental strains SPRD-Cu3 and WKY, and in the two congenic strains, SPRD-Cu3.WKY-Mcstm1 (C5) and SPRD-Cu3.WKY-Mcstm2 (C18). In each strain, each transcript mRNA level of 55-day old control females was set to 1. **A:** NER transcripts; **B:** Checkpoint transcripts; **C:** Cell cycle arrest transcripts; **D:** Apoptosis transcripts. * $p \leq 0.05$.

genes upregulated in our study (*Xpc*, *Cdkn1a*, *Gadd45a*) [48,49]. Expression of these genes might thus be stimulated by *Brca1* overexpression, which is particularly striking in the DBMA-treated WKY rats (Fig. **1B**).

Post-translational modifications ensure a prompt response to DNA injury, but increasing the level of mRNAs involved in the DDR likely provides the damaged cell with an additional means to efficiently repair DNA or to set up an adequate cell response. Interestingly, significant changes in numerous mRNA levels were also observed in the kidneys of carcinogen-treated rats, with lack of cytotoxic or mitogenic effect [50]. Several DDR genes were found to be deregulated, with *Cdkn1a* as the only gene in common with our study. However, these changes were not associated with renal cancer susceptibility.

Among the genes we found to be upregulated in mammary cancer resistant strains, *Pmaip1* (*Noxa*) is the only one that is also located in a mammary cancer susceptiblity QTL (*Mcstm2*) [17]. However, this gene is an unlikely candidate gene, because it acts at a late step of DDR. Furthermore, we found no sequence difference in the coding and promoter regions of the WKY and SPRD-Cu3 gene (data not shown).

DMBA-induced DDR gene activation is present in the mammary cancer resistant strain WKY and also (though somewhat attenuated) in two congenic strains exhibiting partial mammary cancer resistance, thereby demonstrating that both mammary cancer resistance and DMBA-induced DDR gene activation are controlled by the same chromosomes (5 and 18) and suggesting that these two traits might be controlled by the same genes, contained in the *Mcstm1* and *Mcstm2* QTLs. We previously showed that precocious mammary differentiation also segregates with mammary cancer resistance in the congenic strains [16]. A critical feature of precocious mammary differentiation might thus be the ability of mammary cells to generate an efficient DDR, including upregulation of genes involved in this response. We suggest that resistance of the WKY rats to mammary cancer is explained by their capacity to activate an early and robust DDR. This feature also explains our previous observation, namely that DMBA-treated WKY rats do not develop pre-neoplasic lesions [16].

CONCLUSION

Our results show that several genes controlling the DDR are upregulated in the mammary tissue of DMBA-treated mammary cancer-resistant rats, thereby strongly supporting the hypothesis that the DDR is a barrier in early tumorigenesis [28,29].

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