Analysis of genes involved in the PI3K/Akt pathway in radiation- and MNU-induced rat mammary carcinomas

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ABSTRACT

The PI3K/AKT pathway is one of the most important signaling networks in human breast cancer, and since it was potentially implicated in our preliminary investigations of radiation-induced rat mammary carcinomas, our aim here was to verify its role. We included mammary carcinomas induced by the chemical carcinogen 1-methyl-1-nitrosourea to determine whether any changes were radiation-specific. Most carcinomas from both groups showed activation of the PI3K/AKT pathway, but phosphorylation of AKT1 was often heterogeneous and only present in a minority of carcinoma cells. The negative pathway regulator Inpp4b was significantly downregulated in both groups, compared with normal mammary tissue, and radiation-induced carcinomas also showed a significant decrease in Pten expression, while the chemically induced carcinomas showed a decrease in Pik3r1 and Pik3l1. Significant upregulation of the positive regulators Erbb2 and Pik3ca was observed only in chemically induced carcinomas. However, no genes showed clear correlations with AKT phosphorylation levels, except in individual carcinomas. Only rare carcinomas showed mutations in PI3K/AKT pathway genes, yet these carcinomas did not exhibit stronger AKT phosphorylation. Thus, while AKT phosphorylation is a common feature of rat mammary carcinomas induced by radiation or a canonical chemical carcinogen, the mutation of key genes in the pathways or permanent changes to gene expression of particular signaling proteins do not explain the pathway activation in the advanced cancers. Although AKT signaling likely facilitates cancer development and growth in rat mammary carcinomas, it is unlikely that permanent disruption of the PI3K/AKT pathway genes is a major causal event in radiation carcinogenesis.

KEYWORDS: breast cancer, ionizing radiation, MNU, PI3K/Akt pathway

INTRODUCTION

Exposure to ionizing radiation is an established risk factor for the development of breast cancer. Epidemiological studies of the Japanese atomic bomb survivors indicate that the breast is one of the most susceptible organs to radiation-induced carcinogenesis [1, 2]. Other studies on patients exposed to medical radiation provide...
evidence that there is a high risk of second breast cancer among women treated with chest radiotherapy for Hodgkin lymphoma or benign breast disease [3, 4]. However, the molecular mechanisms underlying radiation-induced human breast cancer are yet to be fully characterized, because breast cancer in humans occurs as a result of the combined effects of radiation and other factors such as age, diet, family history, alcohol consumption, and hormone therapy [5].

Animal models are useful for studying the contribution of a single factor in carcinogenesis, such as ionizing radiation and chemical carcinogens. The rat model of mammary tumor is widely used to study breast cancer risk and mechanisms of breast carcinogenesis, because rat mammary tumors are comparable in many aspects to human breast tumors, such as in their high frequency of hormone dependence and pathological processes of malignant transformation; i.e. progression from ductal hyperplasia to ductal carcinoma in situ (DCIS) and carcinoma [6]. Using rat models, mammary tumors induced by ionizing radiation and chemical carcinogens have been shown to exhibit specific genomic aberrations [7]. It has been repeatedly reported that Hras mutations are frequently observed in rat mammary carcinomas induced by exposure to 1-methyl-1-nitrosourea (MNU), but not in radiation-induced carcinomas [8–11]. Loss of heterozygosity (LOH) of several chromosomal regions has been found in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced rat mammary carcinomas [12, 13], whereas LOH is a rare event in carcinomas induced by ionizing radiation and MNU [14, 15]. A study of radiation-induced mammary carcinomas in Sprague–Dawley (SD) rats failed to identify any recurrent loss of DNA copy number [16]. While female SD rats are known to be highly susceptible to spontaneous or radiation-induced mammary carcinomas [17], the F1 hybrids of SD rats and mammary cancer-resistant Copenhagen (COP) rats have a very low incidence of spontaneous mammary carcinomas, yet still have a high incidence of radiation-induced carcinomas. Our earliest investigations with carcinomas from this F1 strain suggest that genes of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway might be potential targets of genomic deletion and/or allele loss (unpublished data).

The PI3K/AKT pathway regulates many cellular functions, such as cell proliferation, growth, survival and motility [18, 19] and is frequently mutated or altered in human cancers [20], including a majority of breast cancers [21–23]. Recently, it has been reported that ionizing radiation induces persistent changes in the expression of genes involved in the PI3K/Akt pathway and causes activation of the pathway in normal rodent mammary glands [24–26], suggesting a link between this pathway and radiation-induced mammary carcinogenesis. Furthermore, our previous investigation revealed that mTOR activity, a downstream target of the PI3K/Akt pathway, was higher in some radiation-induced rat mammary carcinomas than in MNU-induced carcinomas [27].

In the present study, we performed immunohistochemical staining of phospho-AKT1 to assess pathway activation in SD × COP F1 rat mammary carcinomas induced by radiation, with MNU-induced carcinomas analyzed in parallel for comparison. Further, gene expression and sequence analyses for several candidate genes in the PI3K/Akt pathway were examined to investigate the mechanisms underlying pathway activation. Our results suggest that although AKT phosphorylation is a common feature in a subset of cells within rat mammary carcinomas induced by either agent, there are no mutations in key pathway genes or permanent changes to gene expression that correlate with the level of activation in individual carcinomas. Although it is possible that a number of subtle changes might collectively induce AKT phosphorylation, or that activation of the pathway may be important at an earlier stage of carcinoma development, it seems unlikely that radiation-induced alterations of the PI3K/AKT pathway genes are critical events in radiation-carcinogenesis of the rat mammary gland.

MATERIALS AND METHODS

Tumor samples

Animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS). In this study, F1 hybrid rats were created by crossing mammary cancer–susceptible and –resistant rats, Sprague–Dawley (SD) and Copenhagen (COP) rats, respectively. Female SD (Jcl:SD) rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). Male and female COP (COP/Hsd and Iar:COP) rats were obtained from Harlan Sprague Dawley, Inc. (Madison, WI, USA) and the Institute for Animal Reproduction (Ibaraki, Japan), respectively, and were maintained by brother–sister mating. The breeding of the (SD × COP) F1 hybrid rats was performed in our facility at the NIRS. They were maintained under specific pathogen-free (SPF) conditions with controlled temperature (23°C ± 1°C) and humidity (45% ± 5%) under a regular 12 h light–dark cycle. They were fed a standard CE-2 diet (CLEA Japan) and sterile water ad libitum. At 7 weeks of age, female F1 rats (n = 29) were exposed to whole-body γ-radiation (4 Gy) using a 137Cs source (Gammacell; Nordion International, Ottawa, Canada) at a dose rate of 0.5 Gy/min. Female F1 rats (n = 30) received a single intraperitoneal injection of 50 mg MNU/kg body weight at 7 weeks. The treated rats were observed daily, and weekly palpation was started at 2 weeks after the treatments to identify mammary tumors. The animals that showed general deterioration during the observation period (including body weight loss and severe anemia due to tumor development) were euthanized; any mammary cancers were collected, including those discovered post-mortem in rats that were found dead. The characteristics of the carcinomas are shown in Tables 1 and 2. The majority of radiation- and MNU-induced carcinomas (12 of 14 and 10 of 10, respectively) were of luminal subtype. Mammary carcinomas and normal glands from carcinoma-bearing rats were frozen and stored at −80°C.

DNA and RNA sample preparation

Genomic DNA and total RNA were both extracted from the same normal mammary gland and mammary carcinoma frozen samples using the AllPrep DNA/RNA Mini Kit (Qiagen, Tokyo, Japan).

Quantitative RT-PCR

Complementary DNA (cDNA) was synthesized by reverse transcription (ReverTra Ace Toyobo, Osaka, Japan). One sample from an animal found dead and two samples with low RNA yield among the radiation-induced carcinoma samples were not subjected to quantitative expression analysis. Primers were designed for eight genes (Erbb2/HER2, Pik3ca, Pdk1, Akt1, Pten, Pik3r1, Map3k1 and
Table 1. Rat mammary carcinomas induced by ionizing radiation

| Case No. | Tumor weight (g) | Latency period (weeks) | Immunohistochemical score | Subtype | Gene expression fold change | Oncogenes | Tumor suppressor genes | Percentage of p-Akt immunopositive carcinoma cells |
|----------|-----------------|------------------------|---------------------------|---------|-----------------------------|-----------|------------------------|-----------------------------------------------|
| J-2      | 31              | 9                      | 3 3 2 1                  | Luminal A | 1.7 0.91 0.55 0.73          | Erbb2     | Pten                  | 0.39 0.47 0.32 0.33                             |
| J-4      | 9.9             | 11                     | 3 3 1 1                 | Luminal A | n.d. 0.68 1.5 0.65 0.52    | Pik3ca    | Pik3r1                | 0.58 1.4 0.48 0.29                               |
| J-6<sup>a</sup> | 32          | 15                     | 2 0 1 3                | Luminal B | 1.9 1.5 0.88 0.70           | Pdk1      | Pten                  | 0.70 0.39 0.45 0.21                               |
| J-13     | 6.2             | 55                     | 3 2 2 1                | Luminal A | 2.5 1.4 0.87 0.65           | Akt       | Pik3r1                | 1.1 0.45 0.69 0.71                               |
| J-14     | 2.9             | 51                     | 3 2 2 3                | Luminal B | 1.2 0.53 0.39 0.33          | Pten      | Pten                  | 0.35 0.15 0.40 0.13                               |
| J-16<sup>c</sup> | 8.4          | 34                     | 3 2 1 2                | Luminal B | 1.5 1.6 1.0 1.1             | Map3k1    | Inpp4b                | 1.1 1.3 1.6 0.45                                 |
| J-18     | 0.26            | 69<sup>d</sup>         | 3 3 2 1                | Luminal A | n.d. n.d. n.d. n.d.        | Akt       | Pten                  | 1.2 0.53 0.39 0.33                               |
| J-20     | 0.35            | 50                     | 3 2 2 1                | Luminal A | 0.30 0.24 0.80 0.79        | Pten      | Pten                  | 0.59 0.87 1.3 0.19                               |
| J-26     | 2.2             | 71                     | 3 3 2 2                | Luminal B | 0.72 4.4 2.2 1.3            | Map3k1    | Inpp4b                | 0.36 1.6 2.2 0.33                               |
| J-27     | 0.84            | 54                     | 3 2 1 3                | Luminal B | 3.2 0.27 1.0 0.63          | Pten      | Pten                  | 1.5 1.6 0.84 0.70                               |
| J-32     | 1.5             | 83                     | 3 3 2 1                | Luminal A | 1.8 1.3 1.7 1.7            | Map3k1    | Inpp4b                | 1.3 1.7 0.66 0.42                               |
| J-36     | 0.98            | 61                     | 0 0 1 1                | Triple negative | n.d. n.d. n.d. n.d. | Pten      | Pten                  | 1.0 0.99 1.0 0.52                               |
| J-39     | 10              | 85                     | 0 0 1 1                | Triple negative | 1.8 1.3 1.7 1.7 | Map3k1    | Inpp4b                | 0.84 0.70 1.0 0.52                               |
| J-40     | 0.50            | 97<sup>d</sup>         | 3 3 2 2                | Luminal B | n.d. n.d. n.d. n.d.        | Akt       | Pten                  | 1.5 1.6 1.0 0.84                                 |
| Average  | 7.6             | 53                     | 2.5 2.0 1.6 1.6        | n.a.     | (0.88) (1.2) (0.56) (0.40) | Erbb2     | Pten                  | (0.88) (1.2) (0.56) (0.40)                       |

ER = estrogen receptor α, PR = progesterone receptor, HER2 = human epidermal growth factor receptor 2, n.d. = no data, n.a. = not applicable.

<sup>a</sup>Data denote Actb-normalized fold changes relative to matched normal mammary glands.

<sup>b</sup>Sample with a hotspot mutation in exon 9 (codon E542K) of Pik3ca gene.

<sup>c</sup>Sample with an 18-bp deletion in exon 12 (codon 570–575del) of Pik3r1 gene.

<sup>d</sup>Sample that had been nonpalpable until dissection.

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ER = estrogen receptor α, PR = progesterone receptor, HER2 = human epidermal growth factor receptor 2, n.d. = no data, n.a. = not applicable.
Table 2. Rat mammary carcinomas induced by MNU

| Case No. | Tumor weight (g) | Latency period (weeks) | Immunohistochemical score | Subtype | Gene expression fold change* | Percentage of p-Akt immunopositive carcinoma cells |
|----------|------------------|------------------------|---------------------------|---------|-----------------------------|---------------------------------------------------|
|          |                  |                        | ER  | PR  | HER2 | Ki-67 | Oncogenes | Tumor suppressor genes |                                      |
|          |                  |                        | Actb |      |       |       | Erbb2    | Pik3ca  | Pdk1 | Akt | Pten | Pik3r1 | Map3k1 | Imp4p |                |
| K2811 Mt-1 | 22               | 45                     | 3   | 3   | 2    | 1    | Luminal A | 2.07 | 2.65 | 0.94 | 1.15 | 0.86 | 0.58 | 0.86 | 1.06 | 5.2   |
| K2896 Mt-1 | 3.8              | 52                     | 3   | 3   | 3    | 2    | Luminal B | 3.10 | 0.92 | 0.57 | 1.29 | 0.51 | 0.35 | 0.45 | 0.23 | 9.9   |
| K3019 Mt-1 | 65               | 43                     | 3   | 3   | 3    | 2    | Luminal B | 2.63 | 1.25 | 0.44 | 0.88 | 0.51 | 0.20 | 1.20 | 0.12 | 0.5   |
| K3052 Mt-1 | 0.65             | 53                     | 3   | 3   | 2    | 1    | Luminal A | 2.78 | 1.16 | 0.59 | 1.05 | 1.92 | 0.32 | 1.26 | 0.06 | 29    |
| K3064 Mt-1 | 8.1              | 71                     | 3   | 3   | 2    | 1    | Luminal A | 6.37 | 3.70 | 1.04 | 1.60 | 1.03 | 1.24 | 3.74 | 0.37 | 29    |
| K3113 Mt-1 | 0.88             | 74                     | 3   | 3   | 3    | 3    | Luminal B | 2.35 | 1.83 | 0.97 | 2.59 | 0.85 | 0.92 | 4.96 | 0.51 | 20    |
| K3154 Mt-1b| 18               | 54                     | 0   | 3   | 2    | 1    | Luminal A | 1.55 | 0.84 | 0.38 | 0.69 | 0.34 | 0.27 | 1.32 | 0.16 | 3.3   |
| K3154 Mt-2 | 2.3              | 54                     | 3   | 3   | 2    | 1    | Luminal A | 6.72 | 2.34 | 0.87 | 1.12 | 0.76 | 0.83 | 1.81 | 0.42 | 18    |
| K3158 Mt-1c| 2.0              | 54                     | 3   | 3   | 3    | 1    | Luminal A | 3.85 | 1.30 | 0.57 | 0.98 | 0.63 | 0.31 | 1.78 | 0.37 | 9.7   |
| K3160 Mt-1 | 0.76             | 62                     | 3   | 3   | 3    | 1    | Luminal A | 19.14 | 2.61 | 0.83 | 1.56 | 0.67 | 2.72 | 2.08 | 0.76 | 56    |
| Average   | 12               | 56                     | 2.7 | 3.0 | 2.5  | 1.4  | n.a. | 5.1   | (5.2) | (0.94) | (0.24) | (0.54) | (0.44) | (0.76) | (1.4) | (0.31) | (17) |

ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor 2, n.a. = not applicable.

*Data denote Actb-normalized fold changes relative to matched normal mammary glands.

*Sample with a hotspot mutation in exons 6 and 19 (codons S405F and D939G, respectively) of Pik3ca gene.

*Sample with a hotspot mutation in exon 9 (codon E542K) of Pik3ca gene.
Inpp4b), using the Primer Express 3.0 software (Applied Biosystems), and were validated to amplify a single product of the correct size for each gene by agarose gel electrophoresis. The primer sequences and PCR conditions are provided in Supplementary Table S1. PCR was performed using the Stratagene Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) and SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan). The expression level of the target genes was normalized to that of Actb. Expression data from one matched normal mammary gland among the radiation-induced carcinoma samples was excluded from further analysis due to poor amplifiability of Actb, which was more than 100-fold lower than the median value.

Sequence analysis

Mutations in all exons of Pten, Pik3ca, Pik3r1 and Map3k1 genes were screened initially by direct sequencing of PCR products from cDNAs from the twelve radiation- and ten MNU-induced carcinoma samples with both DNA and RNA available, and identified mutations were confirmed by direct sequencing of PCR products amplified from genomic DNA on an Applied Biosystems 3130 Genetic Analyzer using a BigDye Terminator kit (Life Technologies Inc., Carlsbad, CA, USA). The primer sequences and PCR conditions are provided in Supplementary Table S2. All sequences were obtained in both directions. Sequence analysis was done using the ATGC and GENETYX software (Genetyx Corporation, Tokyo, Japan). An in-frame deletion in Pik3r1 in one carcinoma was confirmed by TA cloning of PCR products amplified from genomic DNA, and subsequent sequencing (TOPO TA cloning kit, Invitrogen, Life Technologies, Tokyo, Japan).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue sections (~3 µm thick) were immunostained with primary antibodies against estrogen receptor α (ER) (6F11, Leica Microsystems, Tokyo, Japan); progesterone receptor (PR) (PR-2C5, Invitrogen, Carlsbad, CA); human epidermal growth factor receptor 2 (HER2) (A0485, DAKO Japan, Tokyo, Japan); Ki-67 (M7248, DAKO Japan, Tokyo, Japan); or phospho-T308-Akt1 (ab66134, Abcam, Cambridge, UK), as described previously [28]. Briefly, sections were deparaffinized and rehydrated, and antigen retrieval was performed with autoclave treatment at 120°C in 10 mM sodium citrate buffer (pH 6.0) for 20 min for ER, PR, HER2 and Ki67 and with microwave treatment at 98°C for 20 min for phospho-T308-Akt1. After blocking endogenous peroxidase and non-specific binding sites, specimens were incubated at 4°C overnight for ER, PR, HER2 and phospho-
T308-Akt1 or at room temperature overnight for Ki-67. Then, sections were treated with a peroxidase-conjugated secondary antibody (HistoSimple Stain MAX-PO, Nichirei, Tokyo, Japan). Immunoreaction was visualized by using the 3,3′-diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin. The intensity of staining was graded from 0 to 3 for ER and PR (0 = <1% of all cells with nuclear staining; 1 = 1–9%; 2 = 10–20%; 3 = >20%) and for Ki-67 (0 = <5% of all cells with nuclear staining; 1 = 5–9%; 2 = 10–20%; 3 = >20%), according to published studies [29, 30]. The intensity of membrane expression was graded from 0 to 3 for HER2 according to the DAKO-score. Cancer subtype was also determined according to the St Gallen consensus [31]. To calculate the percentage of phospho-AKT1–positive carcinoma cells, stained sections were scanned using a NanoZoomer 2.0-HT slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with the Definiens Tissue Studio 3.0 software (Definiens, Munich, Germany). A carcinoma sample containing >10% of phospho-AKT1–positive

**Fig. 3.** Expression levels of 8 genes involved in the PI3K/Akt signaling pathway in radiation-induced rat mammary carcinomas. (A) Diagram of the PI3K/Akt signaling pathway. Genes which function as oncogenes in the pathway are indicated by rectangles, and genes which function as tumor suppressors are indicted by dotted rectangles. (B) The relative mRNA expression levels of Erbb2, Pik3ca, Pdk1, Akt1, Pten, Pik3r1, Map3k1 and Inpp4b genes in radiation-induced mammary carcinomas and matched normal mammary glands are shown. N = normal tissues (n = 8); C = carcinomas (n = 10). Ten carcinomas (C) that developed in eight rats were compared with eight matched normal mammary glands (N). The data are presented as box plots. The median values are indicated with horizontal bars in the boxes. The boxes contain the values between the 25th and 75th percentiles. The whiskers extend to the highest values excluding outliers. The circles represent extreme values. Comparison between two groups was done using the non-parametric Mann–Whitney U-test. *P < 0.05; **P < 0.01.
carcinoma cells in the tissue was considered as positive for phospho-AKT1 [32].

Statistical analysis
Statistical analysis was carried out with the StatMate software version 3.0 (ATMS, Tokyo, Japan). Comparison between two groups was done using a non-parametric Mann–Whitney U-test. P < 0.05 was considered to be statistically significant.

RESULTS
Activation of the PI3K/Akt signaling pathway
First, we assessed activation of the PI3K/Akt pathway in the mammary carcinomas (Figs 1 and 2), where we observed that 79% (11 out of 14 cases) of radiation-induced carcinomas and 50% (5 out of 10 cases) of MNU-induced carcinomas were positive for phospho-AKT1 (Tables 1 and 2). Some of the carcinomas showed heterogeneous staining patterns for phospho-AKT1, indicating heterogeneous carcinoma cell populations (Figs 1E and F and 2E and F). In order to determine the cause of the pathway’s activation, we measured the gene expression level for key genes in the PI3K/Akt signaling pathway, comparing against age-matched normal mammary gland tissues. Looking at the negative pathway regulators, the expression of Pten was significantly decreased in radiation-induced carcinomas (Fig. 3), while the decrease was not significant for MNU-induced carcinomas; Pik3r1 levels were significantly lower only in MNU-induced carcinomas (Fig. 4), and Inpp4b levels were decreased in carcinomas of both groups. For the positive regulators, Erbb2 and Pik3ca were both significantly upregulated in MNU-induced carcinomas, while interestingly, Pdk1 was significantly decreased in MNU-induced carcinomas. The phospho-AKT1 staining level was positively correlated with Pdk1 gene expression, but only in the radiation-induced carcinomas (Fig. 5 and Supplementary Figure S1), while positive correlations with Erbb2 and Pik3r1 gene expression levels were observed for MNU-induced carcinomas (Fig. 5 and Supplementary Figure S2). The weak or absent

Fig. 4. Expression levels of 8 genes involved in the PI3K/Akt signaling pathway in MNU-induced rat mammary carcinomas. The relative mRNA expression levels of Erbb2, Pik3ca, Pdk1, Akt1, Pten, Pik3r1, Map3k1, and Inpp4b genes in MNU-induced mammary carcinomas and matched normal mammary glands are shown. N = normal tissues (n = 9), C = carcinomas (n = 10). Ten carcinomas (C) that developed in nine rats were compared with nine matched normal mammary glands (N). The data are presented as box plots. The median values are indicated with horizontal bars in the boxes. The boxes contain the values between the 25th and 75th percentiles. The whiskers extend to the highest values excluding outliers. The circles represent extreme values. Comparison between two groups was done using the non-parametric Mann–Whitney U-test. *P < 0.05; ***P < 0.005.
correlations of the expression of the various regulators with phosphorylation of AKT (as a readout of pathway activation) may be an indication of changes at the level of protein function, or subtle changes across a number of pathway inputs that can collectively alter the state of the network, rather than perturbation of a single signaling node.

Mutations in the PI3K/Akt signaling pathway
To assess whether any of the genes in the pathway had been activated/inactivated by acquired mutations, we sequenced *Pten*, *Pik3ca*, *Pik3r1* and *Map3k1*, each of which are documented to be mutated in human mammary cancers, plus the *Hras* gene which is known to be mutated at high frequency in chemically induced rat mammary carcinomas [10]. Although *Hras* was mutated in 8 out of the 10 MNU-induced carcinomas (all at codon 12), interestingly, there were no *Hras* mutations in the radiation-induced carcinomas. The same heterozygous missense mutation in *Pik3ca* was identified in one radiation-induced carcinoma (Fig. 6) and one MNU-induced carcinoma (Fig. 7); it is predicted to disrupt the helical domain, with an additional MNU-induced carcinoma harboring two independent missense mutations (in the C2 and kinase domains). An in-frame deletion in *Pik3r1* was identified in a single radiation-induced carcinoma (Fig. 8), while no mutations were identified in *Pten* or *Map3k1* in any of the carcinomas. These are also summarized in Table 3.

DISCUSSION
The products of the *Pten*, *Pik3r1* and *Inpp4b* genes function as negative regulators of PI3K activity, either by controlling the levels of PI(3,4,5)P3, which mediates Akt phosphorylation, or by directly increasing PI3K activity [33]. Consistent with our results, it has been reported that loss-of-function mutations of *Pten* in human breast cancer are much less common than promoter methylation and reduced expression levels [34–37], with reduced expression of *Inpp4b* also reported in human breast cancers [38, 39]. However, no direct

### Fig. 5. Correlation between the percentages of cells immunopositive for phospho-AKT1 and the expression levels of *Pdk1*, *Pik3r1* and *Erbb2* genes in radiation- and MNU-induced rat mammary carcinomas. (A) Correlation between the percentages of carcinoma cells positive for phospho-AKT1 and the expression levels of *Pdk1* gene in radiation-induced carcinomas. Correlation between the percentages of carcinoma cells positive for phospho-AKT1 and the expression levels of *Pik3r1* (B) and *Erbb2* (C) genes in MNU-induced carcinomas. *r* = Pearson’s correlation coefficient.

### Fig. 6. A missense mutation in the *Pik3ca* gene detected in a radiation-induced rat mammary carcinoma. (A) Sequence electropherograms of the missense mutation p.E542K (c. g1624a) in exon 9 of the *Pik3ca* gene in a mammary carcinoma. Top, normal mammary gland; bottom, mammary carcinoma (case no. J-6). The nucleotide and polypeptide changes in the carcinoma are shown by red letters. aa = amino acids, nt = nucleotides. (B) Exon and domain structures of the *Pik3ca* gene. The missense mutation was located in exon 9, encoding the helical domain, of the *Pik3ca* gene. p85b = p85-binding domain, RBD = RAS-binding domain, C2 = C2 domain, Helical = helical domain, Kinase = kinase domain.
correlation was observed between the staining levels of phospho-AKT1 and gene expression levels of *Pten* and *Inpp4b* in radiation- and MNU-induced rat mammary carcinomas, suggesting that while their downregulation may permit AKT activation, it does not explain the activation of the pathway. Consistent with a previous report [40], our results suggest that increased *Erbb2* gene expression might contribute to AKT phosphorylation, but this was only evident in the MNU-induced carcinomas, while a contribution from *Pdk1* expression was only observed in radiation-induced carcinomas. However, these correlations were both heavily influenced by one or two carcinomas with both high phospho-AKT1 staining and high *Erbb2/Pdk1* gene expression, which might indicate a causal relationship in rare specific carcinomas, but little influence in the remaining majority of carcinomas. Such a mechanism is consistent with the counter-intuitive positive correlation of *Pik3r1* with phospho-AKT1 staining, where dysregulation of the signaling network in a single carcinoma may result in anomalous expression of a negative regulator while still resulting in pathway activation. Where there are a large number of positive and negative signaling inputs, it is possible that no single protein will show a strong direct influence on the activation of the pathway, since subtle changes of both positive and negative regulators may collectively influence the phosphorylation of AKT, and/or individual carcinomas may utilize different routes to pathway activation [41] that are thus not generalizable across a group of carcinomas, making it difficult to identify statistically significant interactions.

The in-frame deletion detected in *Pik3r1* is predicted to disrupt the amino-acid residues from 572 to 600 in the p85α regulatory subunit of PI3K [42], which inhibits PI3K activity, a mutation also found in human breast cancers [43, 44], though overall, mutation of *Pik3r1* is rare [45, 46]. The ES42K mutation in the *Pik3ca* gene observed in the radiation- and MNU-induced mammary carcinomas is a prominent hotspot mutation leading to constitutive activation of AKT in human breast cancer [47], with *Pik3ca* mutations more prevalent in human breast cancer overall [45, 48] despite being rare in these groups of rat mammary carcinomas. Yet, the small number of carcinomas with mutations in these PI3K/AKT pathway genes did not show any more pathway activation than the carcinomas without mutations, in some cases quite the opposite, casting doubt on the importance of these acquired mutations. One explanation is that activation of the PI3K/AKT pathway may facilitate a particular step in mammary carcinoma development or growth, and that once this barrier is passed, ongoing signaling through this pathway may become redundant. This would have the effect of disconnecting the final phospho-AKT1 levels in the late-stage carcinoma from the state of the network at the critical stage. In many of the carcinomas, the phospho-AKT1 staining was highly heterogeneous, which may also interfere with the correlation of the various signaling proteins in DNA/RNA from a mixed tissue sample. Comparison against gene expression levels in normal mammary tissue that was not exposed to either radiation or a chemical carcinogen might also have revealed potential gene expression changes induced by these agents in non-cancer tissues.

Together, these data suggest that while AKT phosphorylation was a common feature in a subset of cells within rat mammary carcinomas induced by radiation or a chemical carcinogen, this was not usually mediated by either disrupted function by mutation of relevant signaling proteins nor correlated with the final expression levels of these same key genes at the advanced cancer stage, although general increases/decreases could be observed when compared with normal mammary tissue. Our findings suggest that while AKT phosphorylation within carcinoma cells may contribute to cancer growth in particular cases, it is not a major underlying causal event mediated by permanent alteration of the signaling pathway genes;
nor does it appear to be more likely to be involved in mammary carcinomas induced by exposure to radiation compared with induction by a canonical chemical carcinogen. Understanding the mechanisms that underlie the induction of breast cancer following exposure to radiation is essential for future efforts to minimize risk and develop effective countermeasures.

**SUPPLEMENTARY DATA**

Supplementary data are available at the *Journal of Radiation Research* online.

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### CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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