Published in final edited form as:

Cancer Lett. 2010 October 28; 296(2): 186–193. doi:10.1016/j.canlet.2010.04.011.

Chk2*1100delC Acts in Synergy with the Ron Receptor Tyrosine Kinase to Accelerate Mammary Tumorigenesis in Mice

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Abstract

The CHEK2 (Chk2 in mice) polymorphic variant, *CHEK2**1100delC, leads to genomic instability and is associated with an increased risk for breast cancer. The Ron receptor tyrosine kinase is overexpressed in a large fraction of human breast cancers. Here, we asked whether the low penetrance *Chk2**1100delC allele alters the tumorigenic efficacy of Ron in the development of mammary tumors in a mouse model. Our data demonstrate that Ron overexpression on a *Chk2**1100delC background accelerates the development of mammary tumors, and shows that pathways mediated by a tyrosine kinase receptor and a regulator of the cell cycle can act to hasten tumorigenesis *in vivo*.

Keywords

MST1R; Met receptor; breast cancer; receptor tyrosine kinase; hepatocyte growth factor-like protein (HGFL); CHEK2

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women and is the second leading cause of cancer-related mortality in the United States (ACS, 2009). Several susceptibility alleles of genes that can contribute to breast cancer have been identified to date and the majority of these genes have roles in cell cycle and maintenance of genomic integrity [1]. A specific variant of the cell cycle checkpoint kinase 2 (*CHEK2*) gene, *CHEK2**1100delC, in which a C is deleted at nucleotide position 1100, confers a cumulative breast cancer risk of

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Conflict of Interest

The authors do not have any financial or personal relationships with other people or organizations that could inappropriately influence the studies in this report.

37% by age 70 in individuals with a family history of breast cancer compared with an overall 8–10% lifetime risk independent of family history [2,3]. The *CHEK2**1100delC variant is an intermediate penetrance susceptibility allele occurring in approximately 5% (55/1071) [4] to 11% (27/237) [5] of individuals with a family history of breast cancer. Although sequencing for *CHEK2**1100delC in high-risk individuals with family histories of breast cancer is not routinely performed, such screening is now available [6,7].

In mice, Chk2 acts to protect genomic integrity and is activated downstream of ATM. Following DNA damage, Chk2 is phosphorylated on key residues (Ser62, Thr68, Ser73, Thr383, Thr387, Ser516) that result in its activation and sustained kinase activity [8,9]. Chk2 phosphorylates the phosphatases Cdc25A and Cdc25C resulting in Cdc25A degradation and Cdc25C sequestration in the cytoplasm [10]. The activities of Cdk2/CyclinA/E and Cdk1/CyclinB are then inhibited by their persistent phosphorylation status, which results in cell cycle arrest in G1/S and G2/M cell [10,11]. These functions of Chk2, however, may be redundant as these molecules are also targets of the cell cycle checkpoint kinase Chk1 [12]. Also in response to DNA damage, Chk2 can phosphorylate p53 and may interfere with G1 progression and induce apoptosis [13–15]. Recently, a mouse model of the human *CHEK2**1100delC variant was generated by Bahassi *et al.*, in which the wild-type *Chk2* allele was replaced by the *Chk2**1100delC variant [3]. The deletion of a single C nucleotide at position 1100 results in a premature stop codon at position 366 [3,8] and a truncated Chk2 protein that lacks part of the kinase domain (codon positions 220–486) and the remaining C-terminus. The *Chk2**1100delC mRNA, however, is degraded due to nonsense-mediated degradation (NMD), but the degree to which it is degraded varies in a tissue-specific fashion such that the truncated protein may act as a dominant negative in some tissues [3]. In humans, the 1100delC variant confers susceptibility to breast cancer. In mice heterozygous and homozygous for the *Chk2**1100delC tumors develop principally in hormone dependent tissues and not exclusively in mammary tissue, suggesting that alteration of *Chk2* alone may not be sufficient to induce breast tumorigenesis [3,12]. However, when exposed to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), female mice homozygous for the *Chk2**1100delC allele developed tumors significantly earlier than mice with wild-type *Chk2* [16].

The Ron receptor tyrosine kinase, a member of the Met proto-oncogene family, is the receptor for hepatocyte growth factor-like protein (HGFL) [17–20]. Ron is overexpressed and tyrosine phosphorylated in a large number of human cancers including cancers of the breast, prostate, colon, ovarian, bladder, pancreas, and lung [21–23]. Specifically, Ron is overexpressed in approximately 50% of human breast cancers [21,23,24]. Recent *in vivo* studies demonstrated that Ron is an important oncogene during breast tumorigenesis. In the MMTV-PyMT mouse model of breast cancer, deletion of the tyrosine kinase domain of Ron in MMTV-PyMT mice resulted in delayed tumor formation and decreased metastasis [25,26]. Furthermore, mice that are transgenic for Ron under control of the Mouse Mammary Tumor Virus (MMTV) promoter (MMTV-Ron) overexpress Ron specifically in the mammary epithelium, which is sufficient to induce mammary tumorigenesis in mice with 100% incidence, and metastases in 90% of the animals [27]. These studies establish that Ron can be a causal factor in breast tumorigenesis and metastasis. MMTV-Ron tumors were associated with increased expression of cyclin D1 and c-myc, suggesting that normal cell cycle control and possibly tumor cell genomic integrity may be compromised in these mice [27].

Given that Chk2 plays an important role in the maintenance of genomic stability, and that mouse and human studies both implicate the *Chk2* variant as a risk factor for developing breast cancer, we crossed the MMTV-Ron transgene into mice homozygous for *Chk2**1100delC to ask whether the *Chk2**1100delC allele acts in synergy or interferes with

Ron overexpression in breast tumorigenesis. Mice that overexpress Ron and are homozygous for *Chk2**1100delC develop mammary tumors with a significantly reduced latency compared with mice that overexpress Ron alone. The presence of *Chk2**1100delC in mice that overexpress Ron did not, however, alter the progression of the tumors once formed. Western analysis of γ H2AX, a marker of DNA damage, showed that non-tumor bearing mammary glands from MMTV-Ron;*Chk2**1100delC/delC mice had greater DNA damage, as indicated by the increased level of γ H2AX, as compared to MMTV-Ron mice with wild-type *Chk2*. Thus these studies indicate that the presence of *Chk2**1100delC/delC correlates with both DNA damage and with accelerated onset of mammary tumorigenesis.

2. Materials and Methods

2.1 Transgenic mice

The characterization of transgenic mice overexpressing Ron in mammary gland (MMTV-Ron) has been published [27]. The generation of mice expressing the knock-in *Chk2**1100delC allele has also been published [3]. To generate mice expressing both alterations, *Chk2**1100delC homozygous females in a C57/BL6 background were bred to heterozygous MMTV-Ron male mice in the FVB/NJ background. The resultant animals heterozygous for both *Chk2**1100delC and MMTV-Ron in a mixed genetic background were crossed to generate mice that were heterozygous for MMTV-Ron and either homozygous for the *Chk2* variant or wild-type for *Chk2*, and are designated as MMTV-Ron X *Chk2**1100delC/delC (experimental) or MMTV-Ron (control), respectively. Mammary tumor formation was detected by palpation at least once per week. The age of the mouse in days at the time of the initial tumor detection by palpation was noted. *Chk2**1100delC homozygous females were examined for tumor formation at the time of sacrifice. Animal colonies were maintained and procedures performed under approval of the University of Cincinnati Institutional Animal Care and Use Committee.

2.2 Primary mammary epithelial cell isolation

Primary mammary epithelial cells were isolated from combined mammary glands from two virgin wild-type mice or from two virgin *Chk2**1100delC/delC mice per experiment as described previously [28]. Briefly, inguinal and thoracic mammary glands were dissected and digested with 1mg/ml collagenase (Worthington Biochemical, Lakewood, NJ) and 2mg/ml BSA in DMEM:F12 with shaking at 200 rpm for 2 hours at 37°C. Epithelial fragments were purified by six rounds of 10 second pulse centrifugations to a maximum speed of 1000 g in 5% BSA PBS. The purified primary mammary epithelial cells were plated in 50:50 DMEM:F12 supplemented with 1:100 Fungizone (Gibco Invitrogen, Carlsbad, CA), penicillin-streptomycin, gentamicin, and ITS (insulin-selenium-transferrin); 5% fetal calf serum; and 5 ng/ml EGF (epidermal growth factor).

2.3 Primary mammary tumor cell isolation

Tumor-derived cells were isolated from a single tumor per mouse for each experiment. The isolation and digestion of the tumor tissue was performed essentially as described previously [28], with the exception of omitting the pulse-centrifugation. All cells obtained from the tumor were plated in RPMI media supplemented with 1:100 Fungizone (Gibco Invitrogen, Carlsbad, CA), penicillin-streptomycin, gentamicin, and ITS (insulin-selenium-transferrin); 2.5% fetal calf serum; and 10 ng/ml EGF (epidermal growth factor).

2.4 Cell cycle analysis

Wild-type, *Chk2**1100delC/delC, and MMTV-Ron tumor-derived cells at low passage (≤ 3) were plated in 6-well culture dishes and grown for 48 hours. The cells were then exposed to

10 Gy total gamma irradiation using a $^{137}\text{cesium}$ source. After 12 hours, the cells were collected by trypsinization and fixed in cold 70% ethanol for 15 minutes at -20°C . The cells were stained with 0.1% propidium iodide and 50 $\mu\text{g/ml}$ RNase in PBS and examined for DNA content using a Coulter Epic flow cytometer (Beckman Coulter, Fullerton, CA). Quantification of cell cycle distribution from DNA content data was performed using the ModFitLT program (Becton Dickinson, Franklin Lakes, NJ). The percent of the diploid population of cells in G1, S, and G2/M, as well as the percentage of polyploid cells (8n population), were averaged from three independent experiments. Results are reported as the mean $\pm$ SEM.

2.5 Immunocytochemistry

Primary wild-type mammary epithelial and MMTV-Ron tumor-derived cells were plated at low passage (≤ 3) on chambered slides and grown for 48 hours with complete growth media. One set of cells was then exposed to 10 Gy total gamma irradiation using a $^{137}\text{cesium}$ source. The cells were immediately placed on ice after exposure. Fresh, prewarmed media was replaced on the cells, and cells were incubated for 30 minutes at 37°C . The cells were washed twice in PBS and then fixed in 4% paraformaldehyde for 20 minutes, PBS rinsed, and stained. Control unirradiated cells were treated similarly but without exposure to gamma radiation. The cells were permeabilized by treatment with 0.5 % Triton X-100 for 15 minutes at room temperature. Blocking of non-specific sites was done at 37°C for one hour using 20% bovine serum albumin (BSA) in PBS. Staining was performed using an antibody specific for γH2AX (Cell Signaling Technology, Danvers, MA). Secondary detection was performed using an anti-mouse conjugate of AlexaFluor 568 (Molecular Probes, Eugene, OR) and counterstained with DAPI in mounting medium. An isotype control was used to evaluate non-specific staining. The cells were visualized using a Zeiss microscope (Zeiss, Oberkochen, Germany). A minimum of 190 cells per cell line was quantified, and three different Ron overexpressing mammary tumor cell lines were used compared with wild-type primary mouse mammary epithelial cells. Representative images are shown, and the percent of γH2AX -positive cells out of total cells is reported.

2.6 Protein isolation and analyses

Wild-type primary mouse mammary epithelial cells and Ron overexpressing mammary tumor cells derived from MMTV-Ron mice were lysed in RIPA buffer (50mM Tris, pH 7.4, 1% SDS, 0.5% sodium deoxycholate, 0.5% Triton X-100, 150mM NaCl, 2mM EDTA) containing protease inhibitors (Complete tablets, Roche, Indianapolis, IN) and phosphatase inhibitors (HALT, Pierce, Rockford, IL). Mammary gland tissues from MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice were homogenized and lysed in Laemmli buffer containing protease inhibitors (Complete tablets, Roche, Indianapolis, IN) and phosphatase inhibitors (HALT, Pierce, Rockford, IL), sonicated then centrifuged for 10 minutes at 12,000g to remove cellular debris. Proteins were separated by SDS-PAGE and transferred to either nitrocellulose or PVDF membranes (Millipore, Billerica, MA). Membranes were probed with the following antibodies: anti-Ron β chain (Santa Cruz Biotechnology, Santa Cruz, CA); anti-cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated Ser139 γH2AX clone JBW301 (Millipore, Billerica, MA). Membranes were stripped and reprobed with an anti- β -actin antibody (C4) to evaluate protein loading. Horseradish peroxidase conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch, West Grove, PA. Detection was performed using an enhanced chemiluminescence reagent (ECL Plus, GE Bioscience, Chicago, IL). Quantitation of proteins was performed using ImageQuant Software (GE Healthcare, Piscataway, NJ).

2.7 Tumor latency and kinetics

Animals were examined weekly by palpation for the development of tumors. A Student t analysis, comparing experimental and control mice, was used to determine the mean time for tumor initiation (latency). The kinetics of tumor development was reported by plotting the number of tumor-free mice as a function of time. The curves were subjected to log-rank analysis.

2.8 Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, and 4 μ m sections produced. Sections were stained with Harris hematoxylin and Eosin Y for routine histological examination of tumor architecture and the presence of metastatic foci in lungs and liver. For assessment of proliferation, mice were injected intraperitoneally with 0.1 ml of BrdU solution (Amersham GE Healthcare, Piscataway, NJ) 2 hours prior to sacrifice. BrdU incorporation was detected on fixed tumor tissues using a cell proliferation kit (Amersham GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Cell death was analyzed using the In Situ Cell Death Detection Kit, POD (Roche, Indianapolis, IN) according to the manufacturer's instructions. The number of BrdU- and TUNEL-positive cells per field was counted in three fields per tissue from 3 mice per genotype as is represented as a percentage of total cells. The median percent of BrdU-positive and TUNEL-positive cells was determined by Mann Whitney Rank Sum tests.

2.9 Statistical analyses

Statistical analyses including Student t, log-rank, and tests of proportions were performed using SigmaStat software (SAS Institute, Cary, NC).

3. Results

3.1 Cell lines derived from MMTV-Ron mammary tumors display increased DNA damage compared to normal mammary epithelial cells

To evaluate genomic integrity and checkpoint control mechanisms in cells from mice that overexpress Ron, mammary epithelial cells were derived from dissected mammary tumors from MMTV-Ron transgenic mice. Primary mammary epithelial cell cultures were also purified from wild-type mice and served as normal controls. To establish that cells from MMTV-Ron mice maintained high levels of Ron expression, Western analysis was performed on lysates from low passage wild-type mammary epithelial cells (WT) and from low passage MMTV-Ron tumor-derived cells that overexpress Ron (Figure 1A). Ron overexpressing cells also exhibited increased levels of cyclin D1, c-myc, and β -catenin compared to wild-type mammary epithelial cells (Figure 1A, and data not shown), demonstrating that these cells recapitulate the protein content of the intact tumor, similar to results we previously reported [27].

To examine the genomic integrity of mammary tumor-derived cells that overexpress Ron, the presence or absence of DNA damage was assessed by analysis of γ H2AX at nuclear foci, a measure of double strand DNA breaks [29]. To induce double strand DNA breaks, normal mammary epithelial cells from wild-type mice and Ron overexpressing cells from MMTV-Ron mice were exposed to 10 Gy of ionizing radiation. The cells were harvested 12 hours later and examined for γ H2AX accumulation at foci by immunofluorescence. Representative images are shown in Figure 1B. After irradiation, 100% of wild-type low passage mammary epithelial control cells and mammary cells from MMTV-Ron mice show visible γ H2AX staining. However, with no irradiation, only 13% of wild-type mammary epithelial cells were positive for γ H2AX, whereas 49% of the Ron overexpressing tumor-derived cells were positive for γ H2AX staining ($p < 0.01$). Similar results were obtained with cells derived from

mammary tumors from two other MMTV-Ron mice and from mammary epithelial cells derived from two independent mice that lack the MMTV-Ron transgene. These data suggest DNA damage is present and persists in Ron overexpressing cells.

3.2 Mammary tumor cells from MMTV-Ron mice and *Chk2**1100delC/delC mammary epithelial cells have altered cycle profiles and display some aneuploidy

Since transformed cells often display genomic instability that may be associated with aberrant cell cycle kinetics, we compared by flow cytometry the cell cycle distribution of low passage mammary tumor cells derived from MMTV-Ron mice with low passage mammary epithelial cells derived from wild-type mice (Table I). After irradiation, wild-type and Ron overexpressing cells displayed a decrease in the S phase cell population and a significant increase in cells in G2/M. These results suggest that while cells that overexpress Ron harbor DNA damage, they appear to retain intact G1/S and G2/M checkpoints. There also was an increase in the number of Ron X delC/delC cells that showed an aneuploid DNA content ($>4n$) which is consistent with the observed chromosomal instability and the initiation of tumorigenesis in human epithelial cells [30].

The polymorphic variant *CHEK2**1100delC is associated with increased risk of breast cancer in humans [1,2]. Mouse embryonic fibroblasts (MEFs) isolated from *Chk2**1100delC mice exhibit an increase in cells in G2 and a compromised G1/S checkpoint compared with wild-type control cells, even in the absence of DNA damage, suggesting that *Chk2**1100delC cells have compromised cell cycle checkpoints [3,12]. Since mammary tumors comprise about 15% of all tumors that form in female mice homozygous for *Chk2**1100delC [16], we compared the cell cycle kinetics of early passage mammary epithelial cells from these mice with that of control mammary epithelial cells homozygous for wild-type *Chk2*. The mammary epithelial cells from the *Chk2**1100delC/delC mice had significantly more cells in G2/M and fewer cells in S-phase than did equivalent cells from wild-type mice or from MMTV-Ron mice (Table I). There was no significant difference, however, in the proportion of cells in G1 as previously reported [3], perhaps due to the difference in cell types (mammary epithelial cells versus MEFs). In contrast to MEFs, the *Chk2**1100delC/delC mammary epithelial cells showed very little change in cell cycle distribution following exposure to ionizing radiation (Table I), again indicative of intrinsic differences in cell cycle control between these two cell types.

3.3 Mice that express MMTV-Ron on a *Chk2**1100delC/delC background develop mammary tumors with significantly reduced latency

Given that MMTV-Ron mice develop mammary tumors with a 50% latency time of 40 weeks [27], and female mice homozygous for *Chk2**1100delC develop tumors in hormone responsive tissues [16], including mammary tissue, with a 50% latency time at approximately 83 weeks, we tested whether expressing the MMTV-Ron transgene in a *Chk2**1100delC background would affect the time of onset of tumorigenesis. Tumor latency, expressed as percent tumor-free mice per day, was determined and is depicted in Figure 2. Sixty seven percent of the *Chk2**1100delC/delC female mice in this experiment developed tumors, which is slightly lower than the 85% previously reported [16]. While the overall tumor incidence in our study is slightly lower than previously published and may be due to variations in genetic background between experiments, we would like to note that with respect to mammary tumors, 2/24 *Chk2**1100delC/delC female mice in this study developed mammary tumors, and correspondingly in the previous report by Bahassi et al. [16], 2/25 *Chk2**1100delC/delC mice developed mammary tumors. Moreover, mammary tumors have not been observed out to 365 days in wild-type mice derived from transgenic crosses to obtain the MMTV-Ron mice in a variety of genetic backgrounds. However, our studies cannot exclude the possibility that the mixed genetic background or a modifier loci

introduced by the FVB/N background may influence the incidence of tumor formation in the *Chk2**1100delC mice. By comparison, 100% of the MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice developed mammary tumors. Of particular note is that the latency of mammary tumor formation in the MMTV-Ron X *Chk2**1100delC/delC mice (mean = 224 days) was reduced by 25% compared with that of MMTV-Ron mice (mean = 280 days) and *Chk2**1100delC/delC controls (mean = 603 days) (log-rank analysis; $p < 0.001$).

3.4 MMTV-Ron mice with and without the *Chk2* variant develop mammary adenocarcinomas and metastases

Histology of mammary tumors from MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice showed that tumors from both genotypes were mammary adenocarcinomas, as previously reported for MMTV-Ron mice [27]. The tumors did not qualitatively differ between genotypes (Figure 3A). Cell proliferation and cell death in mammary tumors were analyzed by immunohistochemical detection of BrdU-incorporation and TUNEL staining, respectively. There were no significant differences in BrdU- and TUNEL-positive cells in the tumors from MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice indicating that the rates of proliferation and cell death in mammary tumors were similar between genotypes (Table II). Similarly, neither the frequency nor pathology of lung and liver metastases in MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice were different (Figure 3B and data not shown). These data indicate that although mammary tumors arise much earlier in the MMTV-Ron;*Chk2**1100delC/delC mice, the tumors once formed, grow at similar rates and are of similar histology to the MMTV-Ron tumors.

3.5 Cells from MMTV-Ron *Chk2**1100delC/delC mammary glands have more extensive DNA damage than cells from MMTV-Ron mammary glands

The level of genomic instability may be one contributing factor to the earlier onset of mammary tumors in MMTV-Ron X *Chk2**1100delC/delC mice than in MMTV-Ron mice with wild-type *Chk2*. To test this end, the relative amount of accumulated γ H2AX, a marker of DNA damage, was assessed by Western blot on tissue from non-tumor bearing mammary glands from MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice. While mammary glands from MMTV-Ron X *Chk2**1100delC/delC mice were histologically similar to their MMTV-Ron counterparts [27], the Western blots showed that level of γ H2AX was significantly higher in MMTV-Ron X *Chk2**1100delC/delC glands than in mammary gland cells from MMTV-Ron mice with wild-type *Chk2* (Figure 4). The data suggest that the *Chk2**1100delC further compromises genome stability in mammary glands of mice that overexpress Ron.

4. Discussion

Impairment of DNA damage checkpoints and repair pathways are closely linked to genomic instability and cellular transformation [31]. Inherited mutations in the DNA damage checkpoint and repair pathway genes such as *BRCA1*, *BRCA2*, *ATM*, *TP53*, and *CHEK2* are independently associated with a predisposition to malignancy in humans [32–37]. Although several studies have supported a role for Ron receptor tyrosine kinase overexpression in human cancers [22,23,38], none have addressed its potential involvement in promoting genomic instability nor its ability to cooperate with allelic variants that predispose to breast cancer. Here we have utilized mouse models to ask whether Ron overexpression alone can affect genomic integrity and cell cycle regulation in mammary tumor cells and whether the cancer-predisposing *Chk2**1100delC allele can exacerbate potential instability and contribute to Ron-mediated mammary tumorigenesis.

The Ron overexpressing mammary tumor-derived cells had far higher baseline levels of γ H2AX than did epithelial cells derived from wild-type mice, suggesting that overexpression of the Ron receptor in mammary tumor cells contributes to genomic instability and allows cells to progress through the cell cycle without repair of the damage. It is possible that Ron overexpression may confer a degree of resistance to DNA damage-induced apoptosis, which may also result in the observed genomic instability. However, both wild-type and Ron overexpressing cells accumulated in G2/M following radiation and consequent induced DNA damage. Consistent with previously reported findings with MEFs [16], mammary cells from mice homozygous for the *Chk2**1100delC allele showed an accumulation of cells in G2/M even in the absence of challenge with ionizing radiation, presumably in response to persistent ineffectively repaired DNA damage.

Chk2 can suppress the oncogenic effects of accumulated DNA damage in mice [39]. The *CHEK2**1100delC allele, which lacks kinase activity, is a risk factor for breast cancer and possibly other tumor types in humans [1], and the murine *Chk2**1100delC counterpart predisposes mice to several tumor types, including mammary tumors, with a long latency in hormone responsive tissues [16]. Given that cells from MMTV-Ron mice and from mice homozygous for *Chk2**1100delC display evidence of genomic instability, we crossed MMTV-Ron transgenic mice with homozygous *Chk2**1100delC knock-in mice to determine whether the *Chk2**1100delC variant would alter the rate or frequency with which Ron-induced mammary tumors arise. Our data showed that mammary tumors formed in 100% of the MMTV-Ron mice, similar to previous reports [27], and in 100% of MMTV-Ron mice homozygous for the *Chk2**1100delC allele. Only 8% of the *Chk2**1100delC control mice developed mammary tumors, which is consistent with Ron overexpression as a driving factor for mammary tumor initiation in this model. Importantly, however, the *Chk2**1100delC allele reduced the latency of mammary tumors initiation from MMTV-Ron transgenic mice by 25% compared with that observed in MMTV-Ron mice alone. Taken together with the previous study which showed that the *Chk2**1100delC allele also resulted in quicker mammary tumor formation induced by the chemical carcinogen DMBA, as compared to wild-type controls [16], these data suggest that the *Chk2**1100delC allele renders mammary cells more susceptible to transformation. In support of this, we show that there is increased γ H2AX, as a measure of genomic instability, in mammary glands from MMTV-Ron homozygous for the *Chk2**1100delC allele compared to MMTV-Ron controls, suggesting that without functional *Chk2* the resultant accumulation of DNA damage provides a more susceptible cellular environment for transformation. These data suggest a cooperative effect of Ron overexpression and the *Chk2**1100delC allele to increase genomic instability and hasten mammary tumorigenesis.

One potential point of convergence of the Ron and *Chk2* signaling pathways is increases in Cdc25A, which has been shown to result in genomic instability and tumor formation when overexpressed [40,41]. Although Western analyses showed no difference in Cdc25A levels in mammary glands and tumors from MMTV-Ron mice with and without *Chk2**1100delC (data not shown), it is possible that subtle temporal differences in Cdc25A levels or function may account for at least in part the observed effect on mammary tumorigenesis. Alternatively, the reduced latency might also be the result of delayed or reduced cell cycle arrest induced upon DNA damage. Given that Ron overexpression results in upregulation of multiple cell cycle progression and growth promoting signaling targets including β -catenin, cyclin D1, and c-myc [27], it is possible that with the loss of *Chk2* function, these signals are more effectively able to override DNA damage response pathways and promote mammary tumor formation.

Acknowledgments

This work was supported by Public Health Services Grants CA-100002 (SEW), DK-073552 (SEW), R03 ES015307 (EMB), U01 ES11038 (PJS), R01 ES012695 (PJS), T32-CA59268 (SEM and GMK); Digestive Diseases Research Development Center grant DK-064403 (SEW); the Department of Defense Award DAMD17-02-1-0342 (SEW); and a pilot grant from the University of Cincinnati Barrett Cancer Center (PJS and SEW).

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Figure 1. Cells derived from Ron overexpressing tumors recapitulate the properties of the primary tumor

A) Western analysis was performed on protein lysates prepared from virgin wild-type purified murine mammary epithelial cells (WT) and several independent harvests of MMTV-Ron mouse mammary tumor-derived cells (Ron overexpressing) using antibodies specific for Ron and cyclin D1. Actin served as loading control. **B)** Purified wild-type mouse mammary epithelial cells (WT) and MMTV-Ron mouse mammary tumor-derived cells (Ron Overexpressing) were plated on chamber slides and stained for the presence of γ H2AX with (+) or without (–) exposure to 10 Gy ionizing radiation (IR). Representative merged images DAPI-stained nuclei (blue) and γ H2AX (red) are shown. 200 $\times$

magnification. The percent of cells positive for γ H2AX out of total cells counted is indicated in the upper right hand corner of each group. * $p < 0.01$ (–IR WT compared to –IR Ron overexpressing; t-test analysis).

Figure 2. Reduced mammary tumor latency in MMTV-Ron X *Chk21100delC/delC mice compared to MMTV-Ron mice with wild-type *Chk2***

Transgenic mice with mammary-specific Ron overexpression (MMTV-Ron) (○) or MMTV-Ron mice homozygous for the *Chk2**1100delC mutant allele (MMTV-Ron X delC/delC) (▲) were evaluated for mammary tumor latency. The results are plotted as the percent of tumor-free mice per age of mice in days. The latency of tumor formation in female mice homozygous for the *Chk2**1100delC allele (delC/delC) is also plotted as the percent tumor-free mice per age of mice in days, where mice with (□; n=16) and without (■ n=8) tumors are indicated. Of the *Chk2**1100delC/delC mice that developed tumors, only 2 mice developed mammary tumors. The number of mice evaluated was MMTV-Ron, n=41; MMTV-Ron X delC/delC, n=23; delC/delC, n=24.

Figure 3. MMTV-Ron mice with wild-type and mutant *Chk2* developed mammary adenocarcinomas and metastases

Tumors from MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice were examined histopathologically by hematoxylin and eosin staining. **A)** Representative images of the primary mammary tumor are shown, and were mammary adenocarcinomas. **B)** Histological examination of lung tissues from MMTV-Ron and MMTV-Ron X *Chk2**1100delC/delC mice showed the presence of metastases (black arrows) in both genotypes. Representative images are shown. Scale bar, 200μm.

Figure 4. MMTV-Ron X *Chk21100delC/delC non-tumor bearing mammary glands exhibit increased phosphorylated γ H2AX**

Western analyses were performed on mammary glands from wild-type (n=4), MMTV-Ron (n=4) and MMTV-Ron X *Chk2**1100delC/delC (n=5) mice to determine the levels of γ H2AX, as an indication of genome instability. Actin served as loading control.

Cell cycle distribution of low passage mammary epithelial cells derived from wild-type control (WT), *Chk2**1100delC/delC (delC/delC) mouse mammary glands, and MMTV-Ron mouse mammary tumors that express either wild-type Chk2 (Ron) or *Chk2**1100delC/delC (Ron X delC/delC) with and without ionizing radiation *in vitro*.

Table 1

% Cells	-IR		+IR			
	G1	S	G2/M	Polyploid	G1	S
WT	46 ± 2	36 ± 4	17 ± 5	5 ± 1	33 ± 3	28 ± 9
Ron	53 ± 8	33 ± 4	15 ± 4	3 ± 1	49 ± 10	13 ± 4
delC/delC	44 ± 7	16 ± 2	40 ± 9	2 ± 1	41 ± 7	12 ± 2
Ron X delC/delC	46 ± 2	23 ± 2	31 ± 3**	7 ± 2	38 ± 4	14 ± 1
						48 ± 3*

The mean percentage of G1, S, G2/M, and polyploid cells from three independent experiments are shown ± SEM.

* p < 0.05 by t-test on the percentage of cells in G2/M – IR compared to the percentage of G2/M cells + IR for WT, Ron, and Ron X delC/delC cells.

** p < 0.05 by t-test on the percentage of Ron cells in G2/M – IR compared to the percentage of Ron X delC/delC cells in G2/M – IR.

Table II

Rates of cell turnover in tumors from mammary-specific Ron overexpressing mice (MMTV-Ron) and Ron overexpressing mice homozygous for the *Chk2**1100delC variant (MMTV-Ron X delC/delC).

	MMTV-Ron	MMTV-Ron X delC/delC
Proliferation	54	44
Cell death	18	21

The median number of BrdU-positive and TUNEL-positive cells out of total cells was determined. There was no significant difference in cell proliferation or cell death between MMTV-Ron and MMTV-Ron X delC/delC; Mann-Whitney Rank Sum test.