A germ line mutation that delays prostate cancer progression and prolongs survival in a murine prostate cancer model

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Circulating insulin-like growth factor-I (IGF-I) levels have been shown to be related to risk of prostate cancer in epidemiologic studies. While specific genetic loci responsible for interindividual variation in circulating IGF-I levels in normal men have not been identified, candidate genes include those involved in the growth hormone (GH)–IGF-I axis such as the hypothalamic factors GH releasing hormone (GHRH) and somatostatin and their receptors. To investigate the role of the GH–IGF-I axis on *in vivo* prostate carcinogenesis and neoplastic progression, we generated mice genetically predisposed to prostate cancer (the TRAMP model) to be homozygous for *lit*, a mutation that inactivates the *GHRH receptor* (*GHRH-R*) and reduces circulating levels of GH and IGF-I. The *lit* mutation significantly reduced the percentage of the prostate gland showing neoplastic changes at 35 weeks of age ($P = 0.0005$) and was also associated with improved survival ($P < 0.01$). These data provide an example of a germ line mutation that reduces risk in an experimental prostate carcinogenesis model. The results suggest that prostate carcinogenesis and progression may be influenced by germ line variation of genes encoding signalling molecules in the GH–IGF-I axis.

Keywords: *lit*; TRAMP; prostate carcinogenesis; survival

Recent prospective studies have revealed a positive association between serum insulin-like growth factor-I (IGF-I) and prostate cancer risk (Chan *et al*., 1998; Wolk *et al*., 1998; Shaneyfelt *et al*., 2000; Pollak, 2001; Pollak *et al*., 2004; Renehan *et al*., 2004; Stattin *et al*., 2004). Both genetic and lifestyle factors influence IGF-I levels (Harrela *et al*., 1996). Epidemiologic data showing variation of prostate cancer risk according to IGF-I levels are biologically plausible given that normal prostate epithelial cells (Cohen *et al*., 1991) as well as prostate cancer cells (Culig *et al*., 1994; Pollak *et al*., 1999; Hellawell *et al*., 2002) are IGF-I responsive. While specific genetic loci responsible for interindividual variation in circulating IGF-I levels in normal men have not been identified, candidate genes include those involved in the growth hormone (GH)–IGF-I axis such as the hypothalamic factors GH releasing hormone (GHRH) and somatostatin and their receptors. A mutation (*lit*) in the murine *GHRH-R* gene is associated with a loss of receptor function that leads to low systemic GH and IGF-I levels and to small body size (Jansson *et al*., 1986; Godfrey *et al*., 1993; Lin *et al*., 1993) but does not affect androgen levels (reviewed in Chandrashekar *et al*., 2004).

In this study, we used the transgenic adenocarcinoma of the mouse prostate model (TRAMP) that was developed using a $-426/+28$ bp fragment of the rat probasin regulatory sequence to specifically target expression of SV40 early genes to the prostatic epithelium (Greenberg *et al*., 1995; Gingrich *et al*., 1999; Kaplan-Lefko *et al*., 2003). The changes that occur during prostate carcinogenesis in these mice highly resemble human prostate transformation (Greenberg *et al*., 1995; Gingrich *et al*., 1999; Kaplan-Lefko *et al*., 2003) and take place in the setting of normal serum androgen levels (Wang *et al*., 2004). We generated TRAMP mice (C57BL/6 background) to be homozygous for the *lit* mutation and compared prostate incidence and progression in TRAMP and TRAMP/*lit*/*lit* animals, over a 35-week period.

All TRAMP control mice ($n = 19$) developed prostate cancer and 74% either died prior to the 35-week time point, or had to be killed due to advanced disease, in contrast to only 18% of TRAMP/*lit*/*lit* mice ($n = 17$) (Figure 1a). The difference in survival is significant ($P < 0.01$). All animals surviving to the 35-week time point were weighed and examined for gross organ abnormalities at necropsy. The gross pathology showed that the TRAMP mice displayed gross abnormality of the seminal vesicles and tissue invasion while the TRAMP/*lit*/*lit* mice appeared normal without any gross

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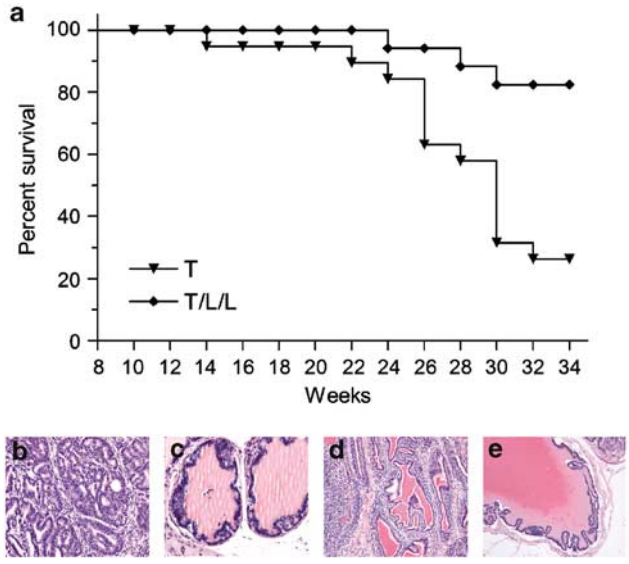

Figure 1 (**a**) Survival of TRAMP mice homozygous for the *lit* mutation as compared to TRAMP controls. TRAMP controls (triangle point, $n=19$) and TRAMP/*lit*/*lit* males (diamond point, $n=17$) were included in experiments and monitored throughout the 35-week period. Survival of TRAMP mice homozygous for the *lit* mutation was significantly greater than that of TRAMP controls ($P<0.01$). (**b–e**) Histopathological sections of the prostate gland and seminal vesicles from TRAMP controls and TRAMP homozygous for the *lit* mutation, the latter showing a higher proportion of non-neoplastic tissue. Hematoxylin and eosin staining of 4-μm thick section cut from paraffin-embedded tissue blocks. (**b**) TRAMP, dorsal prostate scored as primarily invasive cancer (well differentiated) at $\times 200$. (**c**) TRAMP/*lit*/*lit*, Dorsal prostate scored as primarily prostate intraepithelial neoplasia (PIN) at $\times 200$. (**d**) TRAMP, seminal vesicle scored as primarily phylloides-like (hypercellular stroma) at $\times 100$. (**e**) TRAMP/*lit*/*lit*, Seminal vesicle scored as primarily normal at $\times 100$

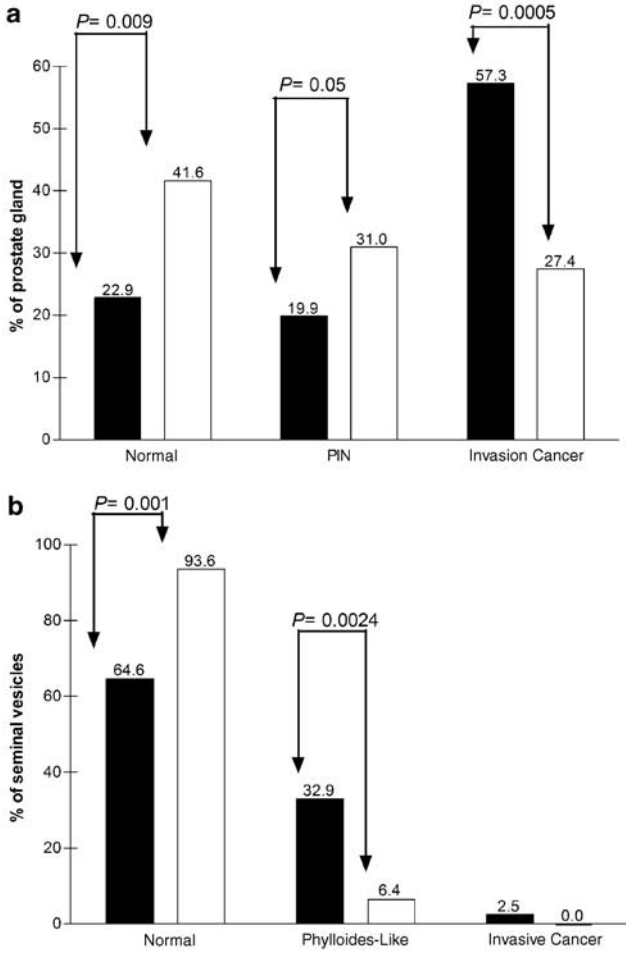

Figure 2 Histopathology scoring according to mouse genotype; TRAMP (black bars) and TRAMP/*lit*/*lit* (white bars). Blinded observers estimated tissue composition of the prostate gland using the following categories: normal, prostate intraepithelial neoplasia (PIN), invasive cancer, or phylloides-like changes, consistent with obstruction. (**a**) Comparison of histopathological scores of whole prostate (mean score of the four lobes of mouse prostate) between genotypes. (**b**) Comparison of histopathological scores of seminal vesicles between genotypes. All values represent mean $\pm$ s.e.m. with *P*-values calculated using Student's *t*-test

abnormality or tissue invasion of the seminal vesicles. We observed that of the five TRAMP mice that survived to that time-point, 100% showed gross evidence of prostate and seminal vesicle neoplasia, in contrast to only 14% of the surviving 14 TRAMP/*lit*/*lit* mice.

As expected, TRAMP/*lit*/*lit* mice were smaller in size than TRAMP mice. The mean body weight for TRAMP/*lit*/*lit* group was found to be 14.26 ± 0.28 versus 30.77 ± 1.24 g of the control group ($P=0.001$). The weight of the genito-urinary organs relative to body weight was reduced in TRAMP/*lit*/*lit* animals as compared to controls (mean ratio 0.04 ± 0.01 versus 0.10 ± 0.01 respectively, $P=0.0019$).

Microscopic examination of the prostate glands revealed a significant impact of the *lit* mutation on prostate neoplasia (Figure 1b and c). Among TRAMP controls, $22.9 \pm 2.2\%$ of the prostate gland at 35 weeks of age was found to be histologically normal, $19.9 \pm 4.5\%$ exhibited prostatic intraepithelial neoplasia (PIN), and $57.3 \pm 5.8\%$ showed invasive cancer, as compared to 41.6 ± 5.5, 31.0 ± 3.4 and $27.4 \pm 4.8\%$ respectively in the homozygous *lit* mice (Figure 2a). The difference between the percentage of the prostate gland with invasive cancer between the TRAMP control and the TRAMP/*lit*/*lit* groups is significant ($P=0.0005$). While the TRAMP mice homozygous for the *lit* mutation had far less invasive cancer at the 35-week time point than the TRAMP controls, we noted a slight increase in PIN in the former as compared to the latter group. This may suggest that the IGF-I deficiency reduced the rate of neoplastic progression from PIN to frank neoplasia.

The histopathology of the seminal vesicles also varied significantly as a function of genotype (Figures 1d, e and 2b). In the TRAMP mice, the seminal vesicles were significantly enlarged while TRAMP/*lit*/*lit* seminal vesicles were normal in size. Among TRAMP control mice, $64.6 \pm 8.8\%$ of the seminal vesicles were scored as normal, as compared to $93.6 \pm 1.9\%$ in the TRAMP/*lit*/*lit* group ($P=0.001$). The presence of invasive adenocarcinoma was rare in the seminal vesicles, and was

confined to the TRAMP controls, where it involved on average $2.5 \pm 2.1\%$ of the tissue. Phylloides-like histopathology, indicative of obstruction, was found to be $32.9 \pm 8.8\%$ in TRAMP controls, but only $6.4 \pm 1.9\%$ in TRAMP/*lit*/*lit* animals ($P = 0.0024$).

As anticipated, TRAMP/*lit*/*lit* mice had lower serum IGF-I and insulin-like growth factor binding protein-3 (IGFBP-3) levels than TRAMP control mice. The mean serum IGF-I levels of TRAMP/*lit*/*lit* measured 19.41 ± 8.64 versus 305.83 ± 81.40 ng/ml in controls ($P = 0.025$). Western ligand blot analysis of serum IGFBP-3 showed a very weak doublet band representing the anticipated low serum levels of IGFBP-3 in the IGF-I-deficient TRAMP mice (data not shown).

As a secondary end point, we used Ki-67 labeling to compare proliferative rate between TRAMP controls and TRAMP/*lit*/*lit* mice in both normal and neoplastic tissue at the 35-week time point. Although we had insufficient tissue for formal quantitative analysis of this end point, Ki-67 staining showed reduced tumor cell proliferation in the TRAMP/*lit*/*lit* animals compared to the TRAMP controls (Figure 3c and d). The difference in proliferation in the untransformed (at-risk) tissue between controls and TRAMP/*lit*/*lit* animals was in the same direction, but less marked (Figure 3a and b).

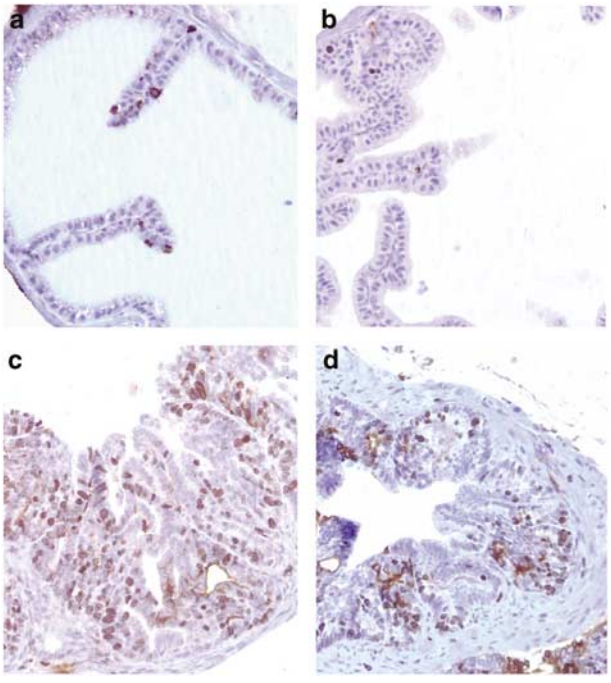

Figure 3 Proliferation of prostate tissue from TRAMP control and TRAMP/*lit*/*lit* mice estimated by Ki-67 staining. Prostate tissue (4 μm-thick section) were stained with anti-Ki-67 rabbit monoclonal antibody, clone SP6 (dilution 1 : 200 for 20 min) (LAB Vision, Fremont, CA, USA). For details, see supplementary methods. (**a**) Ki-67 stain of normal prostate of a TRAMP mouse; (**b**) representative Ki-67 stain of normal prostate of a TRAMP/*lit*/*lit* mouse, showing somewhat lower proliferation than the TRAMP control; (**c**) representative Ki-67 stain of prostate cancer in TRAMP mouse; (**d**) representative Ki-67 stain of prostate cancer in TRAMP/*lit*/*lit* mouse, showing a rather lower proliferation than the cancer in the control TRAMP mouse. Original magnification $\times 200$

Our data identify *lit* as a germ-line mutation that confers protection against prostate carcinogenesis. Although it has been suggested that GHRH-R is present and functional in prostate cells (Chopin and Herington, 2001; Letsch *et al*., 2003), we were unable to detect GHRH-R transcripts in either the normal or the neoplastic prostate tissue of TRAMP or TRAMP/*lit*/*lit* mice (Figure 4). We therefore speculate that in contrast to classic oncogenes and tumor suppressor genes, which act at the cellular level, *GHRH-R* influences carcinogenesis indirectly, acting at the level

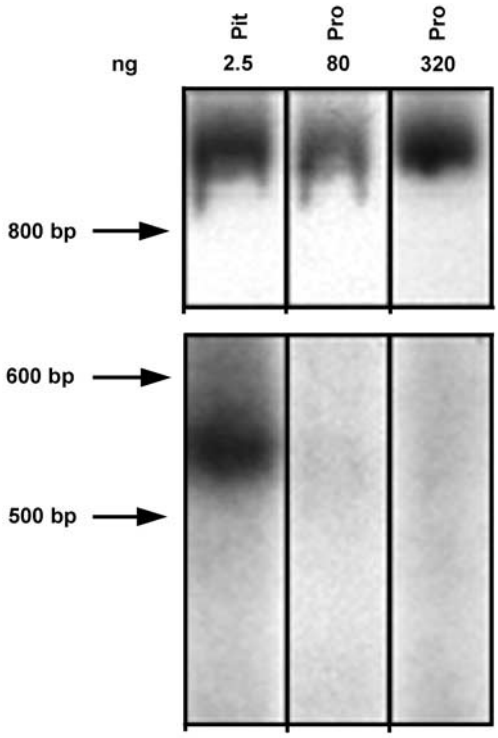

Figure 4 GHRH-R gene expression. Autoradiographic representation of GHRH-R mRNA (575 bp) and GAPDH (836 bp) levels in TRAMP mouse prostate samples compared to C57BL/6 mouse anterior pituitary samples from representative RT–PCR reactions. Total cellular RNA was isolated using the Qiagen Rneasy mini-kit (prostate tissue) (Mississauga, ONT, Canada) and with Trizol (pituitary gland) (Invitrogen/Canada life Technologies, Burlington, ONT, Canada), according to the manufacturer's protocols. Total RNA from mouse prostates (20–320 ng) and anterior pituitaries (2.5 ng) was subjected to one-step RT–PCR using reagents and protocol of the Titan One Tube RT–PCR System kit (Roche Diagnostics, Laval, QC, Canada) using primers for mouse GHRH-R (20 pmol) (Peng *et al*., 2001) and mouse GAPDH (300 pmol) (Aleppo *et al*., 1997). RT–PCR reaction was performed using a Biometra T Gradient PCR (Montreal Biotech Inc., Kirkland, QC, Canada), with the following cycle profile: 30 min at 50.0°C; denaturation at 95.0°C for 3 min, annealing at 66.0°C for 70 s, elongation at 72.0°C for 60 s followed by 39 cycles at 95.0°C for 60 s, at 66.0°C for 70 s, at 72.0°C for 60 s, and a final cycle at 95.0°C for 70 s, 66.0°C for 60 s, and a 5-min elongation step at 72.0°C. GHRH-R and GAPDH PCR products were analysed by gel electrophoresis on 4.5% nondenaturating polyacrylamide gel (for more details see Supplementary methods). In contrast to the anterior pituitary, very low to background levels were observed in prostate samples, even using 320 ng of prostate total RNA for the RT–PCR reaction in TRAMP prostate samples. Pit: anterior pituitary; Pro: prostate

of whole organism by influencing the levels of GH and IGF-I.

While IGF-I signalling has been found to favor survival at the cellular level (O'Connor *et al*., 2000; Pollak *et al*., 2004), activation of the insulin/IGF-I signalling pathway leads to accelerated aging in a variety of model organisms (Guarente and Kenyon, 2000; Kenyon 2001; Dillin *et al*., 2002; Holzenberger *et al*., 2002; Tissenbaum and Guarente, 2002; Arantes-Oliveira *et al*., 2003; Longo and Finch, 2003; Tatar *et al*., 2003; Pollak *et al*., 2004). The *lit* mutation provides an interesting contrast to the recently described $p53^{+/m}$ mutation (Tyner *et al*., 2002). Both are associated with substantial reduction in tumor incidence, but the former leads to enhanced longevity (Flurkey *et al*., 2001) while animals with the latter mutation display an accelerated aging phenotype (Tyner *et al*., 2002).

Higher IGF-I levels may facilitate carcinogenesis and/or early neoplastic progression by increasing the turnover rate of at-risk epithelial cells of the prostate, and/or by decreasing the probability of apoptosis of partially transformed cells. It is of interest that a rare human mutation analogous to the murine *lit* mutation has been described and also causes growth failure (Wajnrajch *et al*., 1996; Maheshwari *et al*., 1998). It is likely, however, that polymorphic variation of genes related to the GH–IGF-I axis has a greater impact on cancer risk and cancer aggressivity in human populations than inactivating mutations. The GH–IGF-I axis may offer novel molecular targets for prevention and treatment of prostate cancer.

Note added in Proof

Drs L Murphy and J Dodd (University of Manitoba) have recently observed that prostate cancer growth in a similar model is reduced when prostate cancer-prone mice are crossed with IGFBP-3 transgenic mice (personal communication).

Abbreviations

IGF-I, insulin-like growth factor I; GH, growth hormone; GHRH-R, growth hormone releasing hormone receptor; IGFBP-3, insulin-like growth factor binding protein-3; TRAMP, transgenic adenocarcinoma of the mouse prostate; PIN, prostatic intraepithelial neoplasia; nt, nucleotide.

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