Generation of Metastatic Variants of Eker Renal Carcinoma Cell Lines for Experimental Investigation of Renal Cancer Metastasis

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We and others have demonstrated that a mutation in Tsc2 is the rate-limiting step for renal carcinogenesis in the Eker rat model. Although inactivation of Tsc2 results in development of renal tumors, it is not sufficient for metastatic renal cell carcinomas (RCs) in the Eker rat. To investigate the additional genetic event(s) necessary for cancer metastasis, we have established highly metastatic S-Lk9d-SLM cell lines from a non-metastatic RC cell line (Lk9dL) by co-implantation with a foreign body (gelatin sponge). Since these cell lines were remarkably different in metastatic performance (all and none, respectively) despite having the same genetic background, they should be useful experimental tools to investigate metastasis-promoting events in renal carcinogenesis.

Key words: Eker rat — Renal carcinoma — Metastasis — Gelatin sponge

Metastasis of human renal carcinomas (RCs) is a serious problem impacting on the prognosis. Therefore, investigation of the underlying molecular mechanism(s) is important. However, because of the genetic diversity in man, it is very difficult to elucidate the rate-limiting steps for metastasis by examination of clinical materials. We have studied renal carcinogenesis in the Eker rat as an example of a Mendelian dominantly inherited predisposition to a specific cancer in an experimental animal.1,2 We and others showed that the responsible gene for Eker RCs is the rat homologue of human tuberous sclerosis 2 (TSC2) gene.3 The phenotype in human tuberous sclerosis (TS) differs from that in the Eker rat, except for the occurrence of RCs (in human, angiomyolipomas are more common),4 although quite recently subependymal and subcortical hamartomas were reported in the Eker rat.5 The Eker rat has great potential for elucidating the role of the Tsc2 gene in renal carcinogenesis. While all heterozygous Eker rats develop RCs through multiple stages from early preneoplastic lesions (e.g., phenotypically altered tubules) to adenomas, these RCs are rarely metastatic. Although “Knudson’s 2-hits” in the tuberous sclerosis gene (Tsc2) have been reported as causative steps for renal carcinogenesis in the Eker rat,6 they are not sufficient in themselves for the genesis of metastatic RCs. Accumulation of genetic alterations is thought to be necessary for the genesis of fully malignant cancers.7 Therefore, some additional mutation(s) must be needed for metastatic modification of RCs in the Eker rat.

To accumulate genetic alterations in Eker RCs, we transplanted cells of an Eker RC cell line together with a fragment of gelatin sponge into subcutaneous tissue of nude mice. Tumor growth acceleration has been reported by co-implantation of cultured cells with foreign bodies such as plastic plates,8 gelatin sponge9 or glass beads.10 Although the precise mechanisms are not fully understood, induction of inflammatory reactive cells and oxygen radicals are thought to be potential causes of this phenomenon. Aliquots of 1×10⁶ Lk9dL cells derived from an RC in an Eker rat2,11 were co-implanted with a 10×5×5 mm section of gelatin sponge (Spongel, Yamano Pharm., Tokyo) into subcutaneous tissue of three BALB/c nude mice (Charles River Japan, Yokohama). One mouse developed a subcutaneous tumor about 1 year after transplantation. A portion of this tumor tissue was excised by surgical operation, and transplanted into other nude mice without any gelatin sponge. Since the metastatic ability of this primary tumor was weak (it required about 6 months to form lung metastatic nodules), a metastatic tumor cell population was selected by means of the in vivo selection method described by Poste and Fidler.12 Selected tumors were placed in primary culture in RPMI 1640 medium to allow establishment of cell lines. Among 23 cell lines thus obtained, no differences in morphology were detected. Interestingly, the parent Lk9dL cells exhibited spindle shapes, whereas the established cell lines showed an epithelial-like morphology in vitro (Fig. 1A). We named these cell lines S-Lk9d-SLM (Spongel-induced Lk9d selected lung metastasis). The parent Lk9dL cells

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The abbreviations used are: Tsc2, tuberous sclerosis gene type 2; PCR, polymerase chain reaction; RCs, renal carcinomas; VEGF, vascular endothelial growth factor.
only grow in collagen-coated dishes and DMEF medium. However, S-Lk9d-SLMs can attach to normal dishes and grow in RPMI 1640.

We next performed polymerase chain reaction (PCR) to confirm the origin of the S-Lk9d-SLMs. Genomic DNAs of Lk9dL, all clones of S-Lk9d-SLMs, normal kidney of an Eker rat and nude mice were examined. Primer sequences for PCR diagnosis were as follows: 5MFJ 5′-ACCATCAGGATGCTGCTGAA-3′, 3MFJ 5′-GAGCACACAAGCAGGCAAG-3′, TSR27 5′-GCGCCAGATTCCCTCATTA-3′. Amplification reactions were performed from 1 µg of genomic DNA in a volume of 25 µl containing 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of dNTP mixture, 1 µM of each primer and 1.25 units of rTaq DNA polymerase.
polymerase (Toyobo, Osaka) in a programmable thermal cycler (Perkin Elmer, Norwalk, CT). The cycling conditions were: 94°C for 30 s (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (synthesis)×35 cycles. Amplified products were separated on 3% agarose gels and visualized by ethidium bromide staining. In Eker rat or cultured cell lines from Eker rat, an inserted sequence exists at intron 30 of one allele of the \textit{Tsc2} gene.\textsuperscript{3} Priming positions in the rat \textit{Tsc2} locus with the primers used are shown in Fig. 1B. Amplification with 5MFJ and 3MFJ primers, resulted in 151 bp products with the rat wild-type \textit{Tsc2} allele, but no products with the inserted allele because of the length of the inserted sequence (about 6.3 kb) at intron 30 (Eker mutation).\textsuperscript{13} With 5MFJ and TSR27 primer amplification, the inserted allele gave rise to 129 bp products, but the wild-type allele was negative because TSR27 specifically targets the inserted sequence. Lk9dL showed products with 5MFJ and TSR27 primers, but not with 5MFJ and 3MFJ, suggesting deletion of the wild-type allele (Fig. 1B, panel b.

![Image](https://example.com/image1.png)

**Fig. 2.** Gross appearance and histology of Lk9dL and S-Lk9d-SLM. (A) Gross appearance. Erythema and crust are apparent at the sites of tumor masses from S-Lk9d-SLMs. (B and C) Histological appearance of tumors induced by Lk9dL (B) and S-Lk9d-SLM (C). (D) Light microscopy of lung of an Lk9dL-transplanted nude mouse. (E) Light microscopy of lung of an S-Lk9d-SLM-transplanted animal. Multiple nodules can be seen. (F) Metastatic nodule of S-Lk9d-SLM cells showing the same histological appearance as the subcutaneous tumor. All histological panels were stained with hematoxylin and eosin.
and c, lane 3), in line with previous results of Southern blotting. All 23 established S-Lk9d-SLM clones showed the same amplification pattern as Lk9dL, with the two primer combinations (representative results for clones 1 and 2 are shown in Fig. 1B panels b and c, lanes 4 and 5). No products were detected from genomic DNAs of nude mice with the two combinations of primers, confirming the specificity of the PCR diagnosis (Fig. 1B, panels b and c, lane 1). Further evidence was provided by direct sequencing of the amplified products with 5MFJ (data not shown). From these results, we can conclude that the S-Lk9d-SLMs were indeed derived from the Eker RC cell line, Lk9dL.

To test for tumorigenicity and metastatic potential, aliquots of 1 × 10^7 cells of the parent Lk9dL line and three randomly selected S-Lk9d-SLM clones (Nos. 1, 19 and 23) were transplanted into subcutaneous tissue of nude mice. Five animals were used for each group. After 6 weeks' observation, all animals were killed under ether anesthesia. Data for mean volumes up to 6 weeks are illustrated in Fig. 1C. All three clones of S-Lk9d-SLM showed a significant increase in tumorigenicity in nude mice as compared to the parent Lk9dL line from 3 weeks after transplantation (P < 0.001: Student's t test). Macroscopically, erythema and crust were observed at the sites of tumor masses of S-Lk9d-SLMs (Fig. 2A). The histopathological appearances of representative subcutaneous tumors derived from Lk9dL and S-Lk9d-SLM cells are shown in Fig. 2, B and C, respectively. While the parent Lk9dL gave rise to sarcomatous tumors, S-Lk9d-SLMs generated adenomatous tumors. The sarcomatous and adenomatous nature of the lesions induced in recipient nude mice reflects the morphological appearance of the Lk9dL and S-Lk9d-SLM cells in vitro.

While Lk9dL did not form metastatic nodules, all clones of S-Lk9d-SLMs formed diffuse lung metastatic nodules in all tested animals at 6 weeks after transplantation (Fig. 2, D and E). Multiple tumor nodules were observed in light microscopic examination of hematoxylin and eosin-stained sections. Intravascular tumor emboli and proliferation and invasion of metastatic tumor cells in the lung were seen (Fig. 2F).

In this study, we report the establishment and characterization of highly metastatic RC cell lines derived from co-implantation of a gelatin sponge fragment with cells of a non-metastatic Eker RC cell line. Although the mechanisms of malignant transformation in the presence of a foreign body are not fully understood, the presence of early-phase inflammatory host-reactive cells and their production of oxygen radicals are possible causes. Inflammation and oxygen radicals are well established to produce DNA damage, mutations and chromosome change. It is not certain whether the progression of our primary tumor was really due to the gelatin sponge or was just a spontaneous malignant transformation, because it only occurred in one out of three animals. However, since Lk9dL has weak tumorigenicity and no large tumor (over 200 mm^3 in volume) developed in three control animals transplanted without any gelatin sponge, the gelatin sponge may have exerted an accelerating influence, as reported previously.

As regards elements known to be associated with cancer metastasis, we compared the expression of vascular endothelial growth factor (VEGF) between parent Lk9dL and S-Lk9d-SLMs by means of northern blotting. However, no difference was detected in VEGF expression between our established high- and low-metastatic tumor cells (data not shown). In addition, the enzyme activities of 68–72 kDa and 92 kDa type IV collagenases were examined by zymogram analysis, but no difference was observed between Lk9dL and several clones of S-Lk9d-SLM (data not shown). Therefore, other unidentified molecules or pathways might be associated with high metastatic performance in S-Lk9d-SLMs, although we cannot exclude the possibility that the high metastatic performance of S-Lk9d-SLMs might just be due to high tumorigenicity.

Our established cell lines (S-Lk9d-SLM) should provide excellent tools for investigation of unknown additional genetic change(s) responsible for metastasis. To isolate metastasis-promoting genes, subtractive analysis of the parent Lk9dL and S-Lk9d-SLM cDNAs by means of the cDNA-RDA method is under way. We have already obtained some clones that are differentially expressed between Lk9dL and S-Lk9d-SLMs. A representative result of northern blotting is shown in Fig. 1D. This gene was dominantly expressed in S-Lk9d-SLM compared to Lk9dL. A database search revealed some homology of this clone with the coding region of retrovirus envelope gene. We have isolated other genes showing differential expression (data not shown) and are now determining their full sequences. We are also conducting a functional investigation of these differentially expressed genes in vitro and in vivo. In particular, we are trying to make transgenic rats with these differential genes, and cross them with Eker rats to ascertain whether metastatic RCs can appear in Tsc2 mutant (Eker) rats.

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