Tohoku J. Exp. Med., 1999, **189**, 87-93

K-Ras Point Mutations in Spontaneously Occurring Endometrial Adenocarcinomas in the Donryu Rat

Koji Tanoguchi, Nobuo Yaegashi, Kumiko Jiko, Akihiko Maekawa,[1] Shinji Sato and Akira Yajima

Department of Obstetrics and Gynecology, Tohoku University School of Medicine, Sendai 980-8574, and [1]*Department of Pathology, Sasaki Institute, Tokyo 101-0062*

Tanoguchi, K., Yaegashi, N., Jiko, K., Maekawa, A., Sato, S. and Yajima, A. ***K-Ras*** *Point Mutations in Spontaneously Occurring Endometrial Adenocarcinomas in the Donryu Rat.* Tohoku J. Exp. Med. 1999, **189** (2), 87-93 —— The Donryu rat has been found to have a high incidence of spontaneous uterine endometrial carcinomas. Moreover the histologic findings, biological nature and pathogenesis of these rat tumors appear similar to those in humans. To determine if the incidence of *H-* and *K-ras* gene mutations in these rat tumors is similar to that in human endometrial cancers, we isolated DNA samples from 2 atypical hyperplasias, 5 simple or complex hyperplasia without atypia, 9 adenocarcinomas and 7 histologically normal tissues, amplified exons 1 and 2 of the *H-* and *K-ras* genes by PCR and hybridized the products with allele specific oligonucleotide probes. *K-ras* point mutations were observed in 1/2 of the atypical hyperplasia (codon 12: GGT→GTT) and 3/9 of the carcinoma (codon 12: GGT→GAT, GGT →AGT, codon 61: CAA→CAC), while they were not detected in 7 of the normal tissues and in 5 of the simple or complex hyperplasia without atypia. *H-ras* point mutations were not detected in any of these DNA samples. These frequencies in this rat model are similar to those in humans. The absence of *K-ras* mutations from simple and complex hyperplasia tissue samples suggests that these mutations are associated with cytological atypia. Our findings suggest that alterations in the *K-ras* gene may be one of the important initiating event in endometrial carcinogenesis in some of the Donryu rat, like the human. ———— Donryu rat; endometrial carcinoma; ras; point mutation; dot blot hybridization

Carcinogenesis is believed to be a multistep process, involving a progressive series of molecular alterations in the genome. Tumor progression in vivo has been best documented in colorectal neoplasms, which have well-defined premalignant (adenoma) and malignant (adenocarcinoma) stages, each of which is associated

Received May 24, 1999; revision accepted for publication September 27, 1999.

Address for reprints: Nobuo Yaegashi, M.D., Department of Obstetrics and Gynecology, Tohoku University School of Medicine, 1-1 Seiryomachi, Sendai 980-8574, Japan.
e-mail: nyaegashi@gonryo.med.tohoku.ac.jp

with sequentially acquired genetic alterations (Bos 1989; Fearon and Vogelstein 1990).

The development of human uterine endometrial carcinoma may also be associated with a series of somatic mutations that may be responsible for the progression of benign epithelium to less differentiated histologic lesions (hyperplasia) and to carcinoma. Mutational activation of the *K-ras* gene has been detected in endometrial cancers and in premalignant tissues, suggesting that activation of this gene may be involved in the development of this neoplasm. *K-ras* point mutations, however, have been detected in only 10–30% of human endometrial carcinomas (Enomoto et al. 1991; Sato et al. 1991; Mizuuchi et al. 1992; Sasaki et al. 1993; Duggan et al. 1994), a frequency too low to constitute an association between these tumors and *K-ras* mutational changes.

The Donryu rat has been reported to be a good animal model for the development of endometrial carcinomas, in that a large fraction (35%) of these animals develop spontaneous tumors (Maekawa et al. 1994; Nagaoka et al. 1994). In addition, ovarian and vaginal cornification and endocrine imbalances have been observed frequently in Donryu rat with endometrial carcinomas, and metastasis to remote organs occurs in approximately 50 % of these animals. In this model, endometrial hyperplasia, the first histologic alteration in the endometrium, occurs at eight months of age, or about 2 months after persistent estrus, suggesting that an estrogen/progesterone imbalance may play an important role in tumor development (Nagaoka et al. 1990). In the rat model, environmental variability is minimal and genetic variability is much less than in humans. Although *H-* and *K-ras* mutations have been detected in many human tumors, including endometrial cancers, as well as in many animal tumor models, the molecular genetic events that occur in this Donryu rat model have not yet been examined (Maekawa et al. 1994; Nagaoka et al. 1994). Therefore, we examined the mutations of these genes in endometrial cancers of the Donryu rat.

Materials and Methods

Histological analysis

Female Donryu rats (70–120 weeks old) were used in this study. The endometria were fixed with 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histologic classification of rat tumors was performed according to the WHO criteria for human endometrial carcinoma (Kurman 1994).

DNA extraction and PCR

DNA was extracted from 14 normal endometria, 15 hyperplasias, and 14 adenocarcinomas as described by Ito et al (1994). Using specific primers (Table 1), a 116-base pair fragment from *K-ras* exon 1 (containing codon 12), a 152-base pair fragment from *K-ras* exon 2 (containing codon 61), an 82-base pair fragment

TABLE 1. *PCR primers and probes for the H-and* ***K-ras*** *genes*

Gene		Primers	
		Exon 1	Exon 2
K-ras	UP	5′-CCTGCTGAAAATGACTGAGTA-3′	5′-GGTGAATATCTTCAAATGAT-3′
	DOWN	5′-TCGTAGGATCATATTCATCC-3′	5′-CTCCTACAGGAAAGAAGTAG-3′
H-ras	UP	5′-AAGCTTGTGGTGGTGGGCGC-3′	5′-CATGGGGAGACGTGTTTACT-3′
	DOWN	5′-TGGTTCTGGATAAGCTGGAT-3′	5′-CTGTACTGATGGATGTCTTC-3′

	Probes	
Gene	Codon 12	Codon 61
K-ras	GTT GGA GCT GGT GGC GTA GG	GCA GGT CAA GAG GA
	CGT	CCA
	TGT	CTA
	AGT	CAC
	GCT	CGA
	GAT	AAA
	GTT	GAA
H-ras	GGC GCT GGA GGC GTG	AC AGC AGG CAA GAG TA
	GAA	AAA
	GCA	GAA
	GTA	CGA
	CGA	CCA
	AGA	CTA
		CAT
		CAC

from *H-ras* exon 1 (containing codon 12), and a 167-base pair fragment from *H-ras* exon 2 (containing codon 61) were PCR amplified from genomic DNA using a GeneAmpTM DNA Amplification Reagent Kit (Takara, Tokyo). The reaction mixture (100 μl) contained 20 μM of each deoxynucleotide triphosphate, 0.2 μM of each two primer and 0.5 μl AmplitaqTM (Takara). The amplification protocol consisted of 40 cycles of 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, 1 minute of extension at 72°C. PCR products were characterized on 2% NuSieve® (FMC Bioproducts, Rockland, ME, USA) agarose gels stained with ethidium bromide.

Point mutations in codon 13 of the *K-ras* gene and the *H-ras* gene were not examined because they were much less frequently detected in human endometrial cancer tissues (Enomoto et al. 1991, 1993; Ignar-Trowbridge et al. 1992; Mizuuchi et al. 1992).

Dot blot hybridization

PCR-amplified DNA was denatured (95°C for 5 minutes) and applied to a nylon filter (Gene Screen Plus; Dupont). Each filter was prehybridized for 4 hours at 47°C with 5×Denhardt's solution (0.1% [w/v]) each of Ficoll Type 400 [Pharmacia Biotech, Tokyo] , bovine serum albumin [Fraction V; Sigma, St. Louis, MO, USA] and polyvinylpyrolidone), 5×SSC, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA. Oligonucleotide probes labeled with ^{32}P for all possible point mutations at codons 12 and 61 of *H-* and *K-ras*, as well as for the wild type genes (Table 1), were added to the prehybridization solution, and the filters were hybridized overnight. The filters

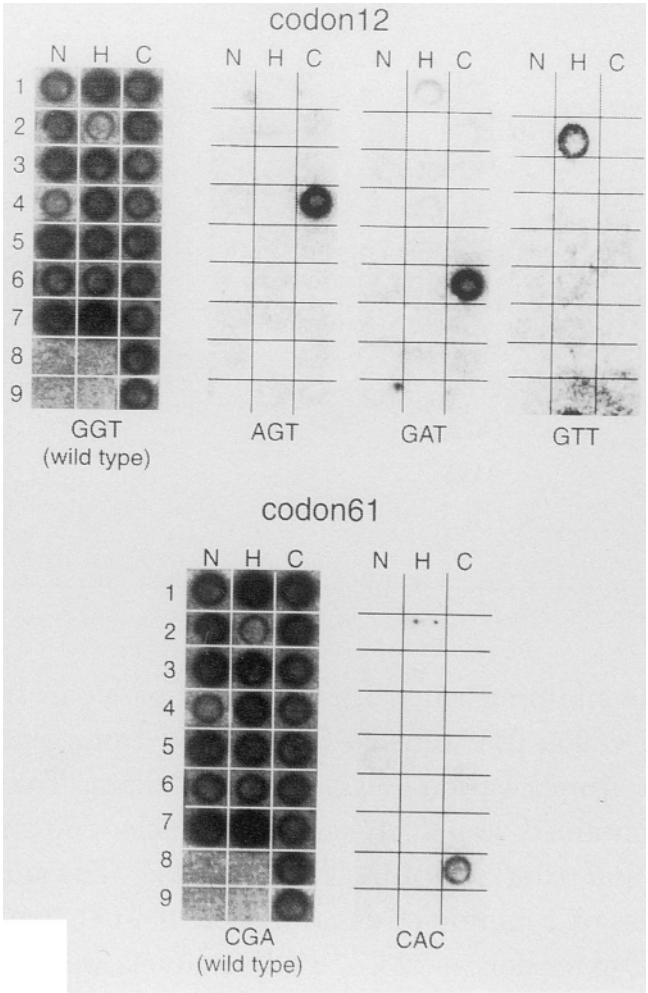

Fig. 1. Detection of point mutations in *K-ras* codons 12 and 61 in Donryu rat endometrial tissues. Exons 1 and 2 of *K-ras* gene were PCR amplified, and mutations were assayed by dot blotting and oligonucleotide hybridization. Point mutations in codon 12 were observed in two carcinomas (C4, C6), and one endometrial hyperplasia (H2) and a mutation in codon 61 was detected in one carcinoma (C8).
N, normal endometria; H, endometrial hyperplasia; C, endometrial carcinomas.

were washed in 2×SSPE, 0.1% SDS for 30 minutes at room temperature and with 3M tetraethyl ammonium, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1% SDS at 56 to 62°C for 30 minutes, and then exposed for 2 to 3 hours to imaging plates, subsequently analyzed with a Bioimage analyzer (FUJI FILM, Tokyo).

Results

We isolated DNA from 14 normal endometrial tissue samples, 15 hyperplasia tissues and 14 adenocarcinomas obtained from Donryu rat uteri, and we were able to amplify *H-* and *K-ras* sequences from 7 normal endometria, 7 endometrial hyperplasias (5 simple or complex hyperplasias without atypia, and 2 atypical hyperplasias), and 9 endometrial adenocarcinomas (Fig. 1). We detected several *K-ras* mutations in these samples, including a GGT→GTT (Gly→Val) point mutation in codon 12 in one atypical hyperplasia, a GGT→GAT (Gly→Asp) mutation in codon 12 in one carcinoma, a GGT→AGT (Gly→Ser) mutation in codon 12 in another carcinoma, and a CAA→CAC (Gln→His) mutation in codon 61 in the third carcinoma. All these mutations substituted an amino acid at the codon. None of these tissue samples had point mutations in codons 12 or 61 of the *H-ras* gene (data not shown). We found that none of the rat tissue samples lacking cytological atypia (0/7 of normal tissues, 0/5 of simple and complex hyperplasia without atypia) had any ras mutations (0/12), while tissues containing atypia (atypical hyperplasia and cancer) had a fairly high incidence ras mutations (1/2 of the atypical hyperplasia and 3/9 of the carcinoma). Statistical analysis by the chi-square test revealed a significant difference ($p<0.05$) in the ras mutation rate between these two groups of samples.

Discussion

Activating point mutations in the ras genes have been demonstrated in many experimental tumor models. In our model of endometrial cancer in the Donryu rat, we detected *K-ras* point mutations in 3 of 9 (33%) adenocarcinomas, and in 1 of 7 (14%) hyperplasias, rates similar to those observed in human endometrial cancers (Enomoto et al. 1990; Sato et al. 1991; Mizuuchi et al. 1992; Sasaki et al. 1993; Duggan et al. 1994). As with the human disease, we were unable to detect *H-ras* mutations in our rat model. While we were able to identify *K-ras* point mutations in atypical hyperplasia, we did not find mutations in normal endometrium, simple hyperplasia or complex hyperplasia without atypia. The absence of mutations in simple or complex hyperplasia suggests that the acquisition of *K-ras* mutations is associated with cytological atypia occurring before malignant transformation. Our results suggests, therefore, that this rat model is genetically similar to human endometrial cancer, even though these animals constitute a relatively homogeneous population in a controlled environment.

Several studies have suggested that there may be two distinct types of human endometrial carcinoma: One occurring from hyperplasia and involving a hor-

monal factor, and another occurring without a hormonal factor and developing by other pathological mechanisms (Silverberg 1980; Bokhman 1983; Deligdish and Holinka 1987). It has been reported that hormonal invariance and hyperplasia occur frequently in the Donryu rat (Nagaoka et al. 1990), although we did not observe *K-ras* gene mutations in the majority of endometrial cancers in these animals. These findings suggest that hormone imbalance, particularly an increased estradiol/progesterone (E2: P) ratio, may results in progressing formation of E2 and estrogen receptor (ER) complex, which works as potent mitogens and initiates tumorigenesis in the process of endometrial carcinogenesis in both humans and Donryu rats. Recently, reported was activation of the estrogen receptor through phosphrylation by mitogen-activated protein kinase (MAPK) in cells transformed by the *K-ras* mutant in the codon 12 (Kato et al. 1995), and enhancement of the steady state level of estrogen receptor by the mutant *K-ras*. These rusults and ours suggest that E2-ER pathway in ras-mediated cell transformation might be important in the initiation event of endometrial cancer (Kato et al. 1997) in both humans and rats.

Acknowledgments

This research was supported in part by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan.

References

1) Bokhman, J.V. (1983) Two pathogenetic types of endometrial carcinoma. *Gynecol. Oncol.*, **15**, 10–17.
2) Bos, J.L. (1989) Ras oncogenes in human cancer: A review. *Cancer Res.*, **49**, 4682–4689.
3) Deligdisch, L. & Holinka, C.F. (1987) Endometrial carcinoma: Two diseases? *Cancer Detect. Prev.*, **10**, 237–246.
4) Duggan, B.D., Felix, J.C., Muderspach, L.I., Tsao, J.-L. & Shibata, D.K. (1994) Early mutational activation of the c-Ki-ras oncogene in endometrial carcinoma. *Cancer Res.*, **54**, 1604–1607.
5) Enomoto, T., Inoue, M., Perantoni, A.O., Terakawa, N., Tanizawa, O. & Rice, J.M. (1990) *K-ras* activation in neoplasms of the human female reproductive tract. *Cancer Res.*, **50**, 6139–6145.
6) Enomoto, T., Inoue, M., Perantoni, A.O., Buzard, G.S., Miki, H., Tanizawa, O. & Rice, J.M. (1991) *K-ras* activation in premalignant and malignant epithelial lesions of the human uterus. *Cancer Res.*, **51**, 5308–5314.
7) Enomoto, T., Fujita, M., Inoue, M., Rice, J.M., Nakajima, R., Tanizawa, O. & Nomura, T. (1993) Alterations of the p53 tumor suppressor gene and its association with activation of the c-*K-ras*-2 protooncogene in premalignant and malignant lesions of the human uterine endometrium. *Cancer Res.*, **53**, 1883–1888.
8) Fearon, E.R. & Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759–767.
9) Ignar-Trowbridge, D., Risinger, J.I., Dent, G.A., Kohler, M., Berchuck, A., McLachlan, J.A. & Boyd, J. (1992) Mutations of the Ki-ras oncogene in endometrial carcinoma. *Am. J. Obstet. Gynecol.*, **167**, 227–232.
10) Ito, K., Watanabe, K., Nasim, S., Sasano, H., Sato, S., Yajima, A., Silverberg, S.G. &

Garrett, C.T. (1994) Prognostic significance of p53 overexpression in endometrial cancer. *Cancer Res.*, **54**, 4667–4670.

11) Kato, K., Ueoka, Y., Kato, K., Hachiya, T., Nishida, J. & Wake, N. (1997) Contribution of enhanced transcriptional activation by ER to [12Val] *K-ras* mediated NIH3T3 cell transformation. *Oncogene*, **15**, 3037–3046.

12) Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T. & Uchiyama, S. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science*, **270**, 1491–1494.

13) Kurman, R.J. (1994) *Blaustein's Pathology of the Female Genital Tract*, 4th ed., edited by Robert K. Kurman, Springer-Verlag, 439–478.

14) Maekawa, A. (1994) Genital tumors in female rats; Influence of chemicals and/or hormones and host factors on their occurrence. *J. Toxicol. Sci.*, **19**, 119–132.

15) Mizuuchi, H., Nasim, S., Kudo, R., Silverberg, S.G., Greenhouse, S. & Garrett, C.T. (1992) Clinical implications of *K-ras* mutations in malignant epithelial tumors of the endometrium. *Cancer Res.*, **52**, 2777–2781.

16) Nagaoka, T., Onodera, H., Matsushima, Y., Todate, A., Shibutani, M., Ogasawara, H. & Maekawa, A. (1990) Spontaneous uterine adenocarcinomas in aged rats and their relation to endocrine imbalance. *J. Cancer Res. Clin. Oncol.*, **116**, 623–628.

17) Nagaoka, T., Takeuchi, M., Onodera, H., Matsushima, Y., Ando-lu, J. & Maekawa, A. (1994) Sequential observation of spontaneous endometrial adenocarcinoma development in Donryu rats. *Toxicol. Pathol.*, **22**, 261–269.

18) Sasaki, H., Nishii, H., Takahashi, H., Tada, A., Furusato, M., Terashima, Y., Siegal, G.P., Parker, S.L., Kohler, M.F., Berchuck, A. & Boyd, J. (1993) Mutation of the Ki-ras protooncogene in human endometrial hyperplasias and carcinoma. *Cancer Res.*, **53**, 1906–1910.

19) Sato, S., Ito, K., Ozawa, N., Yajima, A. & Sasano, H. (1991) Analysis of point mutations at codon 12 of *K-ras* in human endometrial carcinoma and cervical adenocarcinoma by dot blot hybridization and polymerase chain reaction. *Tohoku J. Exp. Med.*, **165**, 131–136.

20) Silverberg, S.G., Mullen, D., Faraci, J.A., Makowski, E.L., Miller, A., Finch, J.L. & Sutherland, J.V. (1980) Endometrial carcinoma: Clinical-pathologic comparison of cases in postmenopausal women receiving and not receiving exogenous estrogens. *Cancer*, **45**, 3018–3026.