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Characterization of Mammary Plaques in DDD Mice Congenic for *Mtv-2* Gene, DDD/1-*Mtv-2/Mtv-2*

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DDD/1 mice free from exogenous mouse mammary tumor virus (MMTV) do not develop any neoplastic mammary lesions. In GR mice, the expression of *Mtv-2*, an endogenous proviral MMTV, leads to 100% incidence of mammary ductal hyperplasias and tumors. An *Mtv-2* congenic line, DDD/1-*Mtv-2/Mtv-2*, was established by introducing *Mtv-2* from GR into DDD/1 to elucidate its function. Development of mammary plaques (MPQ) characterized by ductal hyperplasias was investigated in 152 congenic females on day 17 to 19 of the first pregnancy. The incidence of MPQ was 48.0% and most MPQ-positive mice (75.3%) had only one MPQ. Generally, MPQ were small in size: the diameter was as small as ≦3 mm in 77.6% of them. Of 84 MPQ implanted into intact fat pads, 43 (51.2%), 38 (45.2%) and 3 (3.6%) showed undetectable, pregnancy-dependent and autonomous growths, respectively when the hosts underwent pregnancy. Almost all MPQ produced normal-appearing ductal-alveolar outgrowths in mammary epithelium-divested or cleared fat pads of virgins. MPQ implanted into cleared fat pads were very similar to normal mammary glands in the responses to progesterone (P) and estradiol (E) alone or in combination except for association of ductal hyperplasias in 4 of 12 MPQ under E+P treatment. These findings revealed the preneoplastic nature of MPQ. Exogenous MMTV proviruses were demonstrated in all MPQ. The *int-2* DNA rearrangement was found in 2 of 10 MPQ but in none of 9 mammary carcinomas and the *int-1* DNA rearrangement in none of 10 MPQ but in 5 of 10 carcinomas. It is thus likely that the *Mtv-2* gene participates in a very early stage of mammary tumorigenesis not directly but indirectly through insertion mutation of host genes, while the cellular oncogenes, *int-2* and *int-1*, may contribute to preneoplastic transformation of mammary epithelium and progression from preneoplastic to more malignant states, respectively.

Key words: DDD mouse — Congenic for *Mtv-2* — Mammary plaque — Preneoplastic property

Laboratory and feral mice carry more than 20 mouse mammary tumor proviruses (*Mtv*) as a complete or incomplete unit in the germline.[1,2] Intriguingly, recent studies have demonstrated that *Mtv* genes control the expression of the superantigens that cause the clonal depletion and/or anergy of the relevant Vβ-bearing T cells.[3–5] However, what relation this phenomenon has with mammary tumorigenesis remains to be elucidated. From the oncological point of view, the *Mtv-2* gene on chromosome 18 found in the European mouse strain, GR, has a significant role in the high mouse mammary tumor virus (MMTV) expression in the milk and the early pregnancy-dependent mammary tumors of breeding females.[6,7] The hormone-dependent (HD) tumors are considered to arise from disk-like preneoplastic lesions called "plaques," which have a symmetrical organoid structure. Both HD tumors and plaques have been reported in other European mice, RIII, BR6, DD and DDD.[8,9] A very stable, pregnancy-dependent mammary tumor line, TPDMT-4, was obtained in DDD strain, despite the low incidence and late development of mammary tumors.[10,11] The expectation of more frequent development of stable HD mammary tumors motivated us to introduce *Mtv-2* into DDD from GR mice.[12] Thus, congenic DDD-*Mtv-2/Mtv-2* mice were constructed by crosses between DDD females and GR males, followed by 12 backcrosses to DDD mice using mammary tumors as a marker for selection. These mice have the DNA information corresponding to the *Mtv-2* gene in addition to their original *Mtv* genes and develop mammary tumors at an about 80% incidence at one year of age under breeding conditions. The present study was conducted to characterize the mammary plaques which develop during pregnancy in the congenics.

Abbreviations: MMTV, mouse mammary tumor virus; HD, hormone-dependent; C, cholesterol; E, 17β-estradiol; P, progesterone; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Mice DDD/1 (DDD) mice free from milk-transmitted MMTV and DDD/1-*Mtv-2*/*Mtv-2* (DDD-*Mtv-2*) congenics were bred at the Laboratory Animal Research Center, IMSUT and used for experiments.[13] DDD mice served as controls and recipients.

Detection of mammary plaques The mammary plaque is a disk-like lesion which grows during pregnancy and wanes after delivery. Microscopically, it has a symmetrical organoid structure comprising ductal and alveolar-like elements. Macroscopically, the plaque is pink and easily distinguishable from the surrounding normal mammary gland tissue in late-pregnancy mice. Thus, 8- to 10-week-old DDD-*Mtv-2* females were mated with males and killed on day 17 to 19 of pregnancy. Mammary glands were totally exposed and observed for the presence of plaques with the naked eye and under a stereo microscope if required. Plaques were counted, measured (diameters), cut out and used immediately for transplantation, fixed for histology or stored at −80°C for DNA extraction.

Transplantation of plaques One or 2 tissue pieces approximately 1×1×1 mm in size were cut out from individual plaques. Pieces from different plaques were used in each of Experiments 1 to 3. Experiment 1 was conducted to investigate the effect of pregnancy on the tumorous growth of plaques. DDD females aged 8 to 10 weeks received implantation of a plaque tissue piece into the intact right inguinal fat pad and were immediately kept with males to be impregnated until the second pregnancy was confirmed. They were frequently checked for pregnancy and delivery by visual inspection and newborn babies were removed as soon as possible. The implanted sites were palpated twice weekly to detect tumorous growth, and the perpendicular diameters of palpable tumors were measured. The mean of both diameters was called tumor diameter and used to follow the growth of a tumor. Experiment 2 was conducted to observe the outgrowths of plaques in mammary epithelium-divested or so-called cleared fat pads. Hence, a plaque tissue piece was grafted into the right inguinal fat pad concurrently with surgical removal of the mammary rudiment by the method of DeOme *et al.*[14] in 3-week-old DDD females. The animals were kept as virgins and killed 6 months later. The graft-bearing fat pads were examined as wholemounts as described previously.[15] In Experiment 3, the effects of hormones on the outgrowth of plaques were investigated. For this, DDD females underwent surgical removal of the mammary rudiment in the right inguinal fat pad at 3 weeks and bilateral ovariectomy at 5 weeks of age. One week later, they received implantation of a plaque tissue piece into the cleared fat pad and insertion of a 50-mg pellet containing either cholesterol (C) alone or 0.16 mg 17β-estradiol (E), 39.9 mg progesterone (P) or 0.16 mg E plus 39.9 mg P in addition to C under the dorsal skin. The graft-bearing fat pads were also examined as wholemounts 4 weeks after implantation.

Histology Mammary plaques were fixed in 95% ethanol: glacial acetic acid (99:1, v/v), embedded in paraffin, sectioned at 4 μm and used for immunoperoxidase staining of actin, type IV collagen and MMTV antigens (p27 and gp52) as described previously.[16,17] In brief, immunohistochemistry was performed on deparaffinized serial sections using the avidin-biotin system (ABC Vectastain Kit, Vector Laboratories, Burlingame, CA). Sections were incubated with specific antisera for 1 h at room temperature. The substrate for development of the peroxidase activity was DAB (Wako Pharm. Co., Tokyo). For visualization of type IV collagen, sections were pretreated with 0.01% actinase (Kaken Pharm. Co., Tokyo) in 0.01 *M* PBS, pH 7.2, at 37°C for 15 min. Sections were weakly counterstained with Gill hematoxylin. Separately, all the sections were stained with hematoxylin and eosin. In addition, representative wholemount preparations from Experiments 2 and 3 were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin.

DNA analysis High-molecular-weight DNA was extracted from individual plaques. DNA samples were digested to completion with an excess of restriction enzymes and fractionated by electrophoresis on agarose gels. After transfer of DNA fragments from the agarose gels to nitrocellulose filters, specific DNA sequences were detected by molecular hybridizatioin with suitable ^{32}P-labeled probes. *Eco*RI and MMTV-*env* probe were used for detection of MMTV proviruses, *Eco*RI or *Bgl*II and *int-1* probe C for detection of the *int-1* gene[18] and *Eco*RI or *Sac*I and *int-2* probe c, f or j for detection of the *int-2* gene.[19]

Mammary carcinomas from DDD-*Mtv-2* breeders were included for comparison in histology and DNA analysis.

RESULTS

Incidence of mammary plaques The presence, number and size of mammary plaques were examined in a total of 152 late-pregnancy DDD-*Mtv-2* females. As presented in Table I, 73 (48.0%) of them had 1 to 4 plaques (most had only one). Since mammary plaques were generally round in shape, the largest diameter was measured in 85 plaques. Most (77.6%) of them were ≦3 mm in diameter. In particular, 11 (12.9%) were as small as < 1 mm in diameter and discernible to the naked eye. Sixty late-pregnancy DDD mice included as controls were examined in the same fashion without finding any lesions suggestive of mammary plaques.

Table I. Incidence and Sizes of Mammary Plaques in DDD-*Mtv-2* Females on Day 17 to 19 of First Pregnancy

	Observed	Number of plaques/mouse				
		0	1	2	3	4
Number of mice	152	79	55	14	3	1
Percent	100	52.0	36.2	9.2	2.0	0.7

	Measured	Diameter of plaques (mm)					
		~≦1	~≦2	~≦3	~≦4	~≦5	7
Number of plaques	85	29	20	17	12	6	1
Percent	100	34.1	23.5	20.0	14.1	7.1	1.2

Effect of pregnancy on tumorous growth of plaques (Experiment 1) Mammary plaques implanted into intact fat pads were examined for response to pregnancy. They fell into 3 groups in terms of tumorous growth: undetectable, pregnancy-dependent and autonomous. In the first category the grafts did not produce palpable growth at all throughout 2 cycles of pregnancy and delivery, in the second they grew during pregnancy and regressed after delivery with a growth peak immediately before delivery and in the last they continued to grow independently of pregnancy and delivery. Finally, 84 plaques were divided into 43 (51.2%) undetectable, 38 (45.2%) pregnancy-dependent and 3 (3.6%) autonomous growths. The grafts were visible as a small solid mass in 32 but were unidentifiable in 11 of the 43 tumor-free fat pads at autopsy. Generally, the pregnancy-dependent tumors began to grow around day 10 of the first pregnancy, reached a peak at as little as 4 to 5 mm tumor diameter and regressed completely after delivery, followed by a similar growth pattern during and after the second pregnancy.

Outgrowths of plaques in cleared fat pads of virgin mice (Experiment 2) Eighty-one plaques were implanted into cleared fat pads of virgins and observed for tumor formation by weekly palpation for 6 months. Two plaques formed palpable tumors after 10 and 19 weeks, respectively. Hence, 79 fat pads were examined as wholemounts at the end of the observation period and representative outgrowths are presented in Fig. 1. Since only 2 fat pads had a tumorous outgrowth with peripheral ductal-alveolar structures (Fig. 1A), the tumorigenesis rate of plaques was as low as 5.0%. Most of the plaques (74; 91.4%) formed gland-like outgrowths: 2 were hyperplastic (Fig. 1B) and 72 were normal-appearing ductal-alveolar, filling the fat pad completely in 51 (Fig. 1C) and partially in 21 (Fig. 1D). The last 3 (3.7%) did not produce significant outgrowths. Histologically, the hyperplastic outgrowths consisted of large clusters of acini with secretion in the lumina and ductules rich in stromal and fibrous elements, and the ductal-alveolar structures were composed of thick ducts, ductules and small clusters of acini and similar to maximally developed virgin or normal early-pregnancy mammary glands.[15] Thus, the plaque

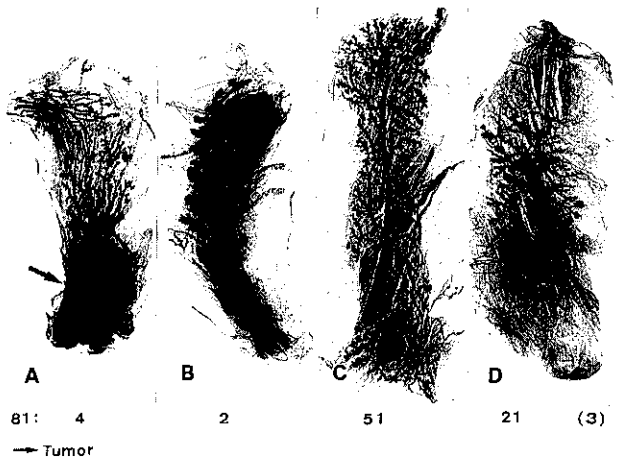

Fig. 1. Outgrowths of mammary plaques from pregnant DDD-*Mtv-2* females in mammary epithelium-free fat pads of DDD virgins. (A) Tumor formation accompanying normal-appearing ductal-alveolar outgrowth at the periphery. (B) Hyperplastic gland-like outgrowth without tumorous foci. (C) Normal-appearing ductal-alveolar outgrowth completely filling the fat pad. (D) Normal-appearing ductal-alveolar outgrowth partially filling the fat pad. Figures at the bottom: Of 81 plaques implanted, 4 developed tumors, 2, 51 and 21 formed similar structures to those in B, C and D, respectively, and 3 (in parenthesis) did not outgrow. See the text for further details. Hematoxylin, ×1.7.

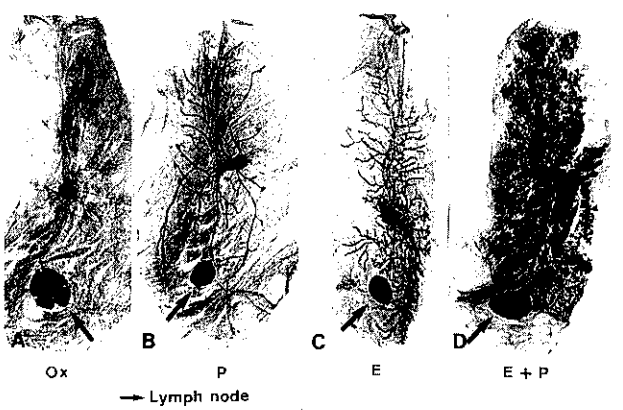

Fig. 2. Effects of hormones on outgrowths of plaques. Plaques were implanted into mammary epithelium-free fat pads of ovariectomized mice. (A–D) Mice were untreated (Ox) or treated with progesterone (P) and estradiol (E) alone or in combination (E+P) for 4 weeks after implantation. See the text for details. Hematoxylin, ×1.7.

appeared very low in tumorigenic potential and more sensitive to the constitutive levels of hormones than the normal mammary gland in virgins.

Effects of hormones on outgrowths of plaques (Experiment 3) Twelve plaques were implanted into cleared fat pads of ovariectomized mice carrying a C, E, P or E+P pellet and examined as wholemounts 4 weeks later. In the C pellet-carrying or untreated controls, 7 plaques did not outgrow at all and the other 5 formed very short, thin ducts (Fig. 2A). In the P-treated mice, 2 plaques did not produce significant outgrowths and the other 10 formed longer and thicker ducts without acini, which differed from one plaque to another in length (Fig. 2B). In the E-treated mice, 1 plaque did not outgrow and the other 11 formed more branched ducts with a few acini which differed from one plaque to another in the degree of expansion into the fat pad (Fig. 2C). All 12 E+P-treated plaques formed hyperplastic mammary gland structures with plaque-like outgrowths in 4 (Fig. 2 D). Histologically, they were more abundant in fibrous tissue and contained less secretion in acinal lumina, but appeared more developed as compared with normal late-pregnant mammary glands.

Histology Mammary plaques were characterized by the branching of duct-like and lobule-like elements, which showed marked intraluminal epithelial proliferation (Fig. 3A). In contrast to the normal mammary gland where actin was strongly stained in the myoepithelial cells, no distinct actin-positive cells were seen in mammary plaques and carcinomas (Fig. 3B). Anti-type IV collagen serum revealed continuous linear staining surrounding epithelial cell nests in both plaques and carcinomas (Fig. 3C). MMTV-gp52 and -p27 antigens were not detected in proliferating cells of plaques, but heterogeneous staining was frequently observed in carcinomas (data not shown).

Detection of MMTV proviruses in plaques DNA isolated from mammary plaques and carcinomas was digested with *Eco*RI and analyzed with MMTV-*env* probe. This enzyme-probe combination reveals each MMTV provirus as a single DNA fragment.[20] As shown in Fig. 4, one or more novel bands (arrowheads) resulting from proviral insertion were seen in addition to the endogenous proviruses including *Mtv-2* (arrow) in all plaques and carcinomas analyzed. This indicated that MMTV proviral insertion might contribute to development of the preneoplastic lesions. However, no insertion sites were common to all plaques and carcinomas.

DNA rearrangements at *int* loci in plaques Activation of two cellular oncogenes, *int-1* and *int-2*, by MMTV pro-

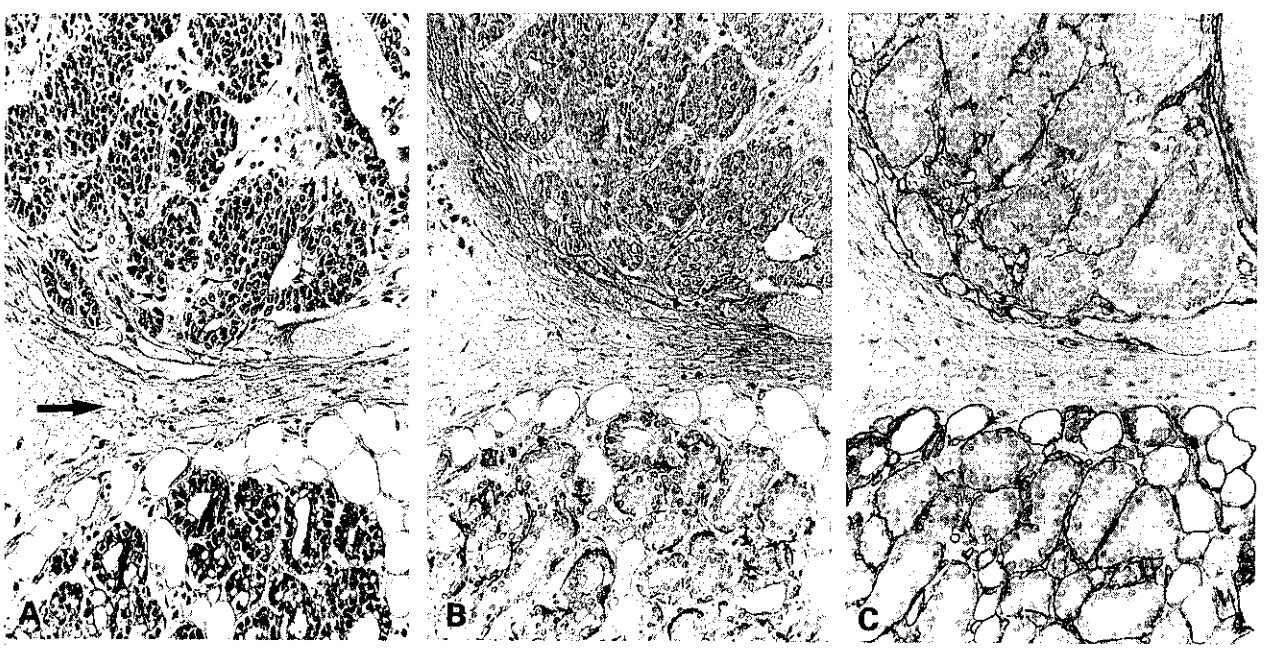

Fig. 3. Histology of plaques. (A) Plaque (upper part) composed of ductal hyperplasias is separated from normal gland (lower part) by abundant fibrous connective tissue (arrow). Note active cell proliferation resulting in partial to complete occlusion of ductal lumina. Hematoxylin and eosin, ×200. (B) Immunohistochemical staining of actin, ×200. Note strong staining of myoepithelial cells in normal gland (lower part) and the absence of distinct actin-positive cells in plaque (upper part). (C) Immunohistochemical staining of type IV collagen, ×200. Note continuous linear staining surrounding cell nests in plaque (upper part) and ducts in normal gland (lower part).

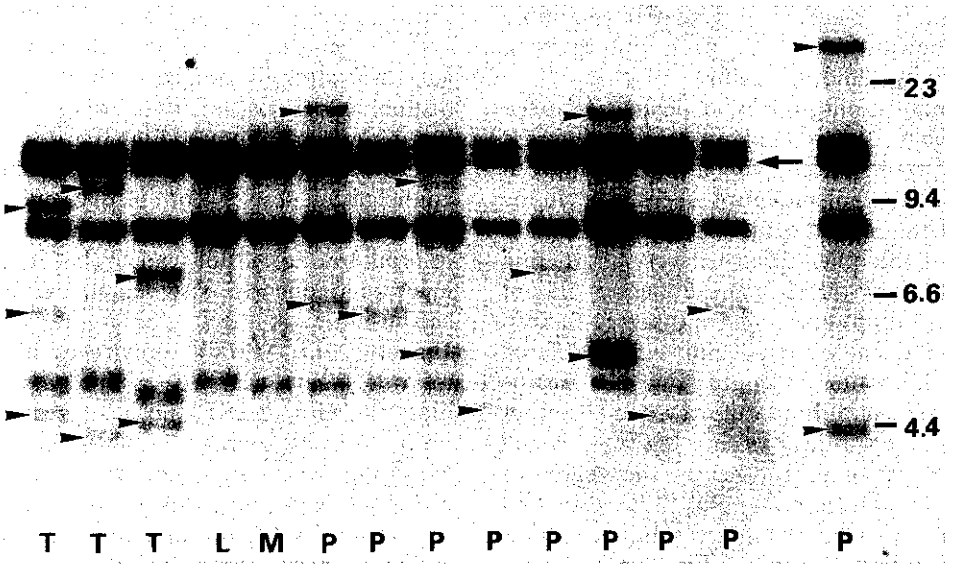

Fig. 4. Southern blot analysis with *Eco*RI and MMTV-*env* probe of DNA from liver (L), normal mammary gland (M), and mammary plaques (P) and carcinomas (T) of DDD-*Mtv-2* mice. The arrow indicates *Mtv-2* DNA fragment and arrowheads indicate exogenously integrated MMTV provirus DNA fragments. Bars on the right are molecular weight markers in kb.

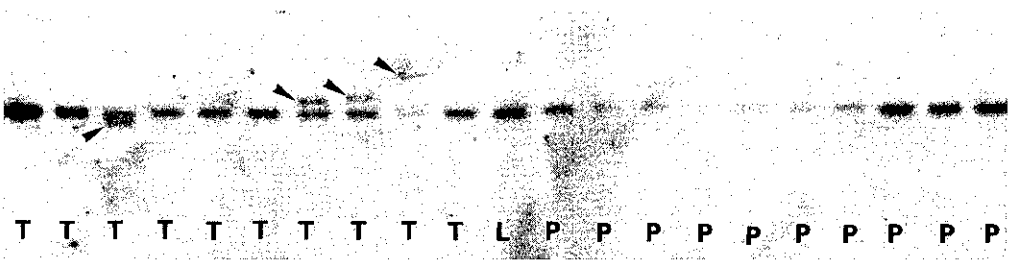

Fig. 5. Southern blot analysis with *Bgl*II and *int-1* probe C of DNA from liver (L), plaques (P) and carcinomas (T) of DDD-*Mtv-2* mice. Arrowheads indicate novel restriction fragments of *int-1* gene resulting from MMTV proviral insertion.

viral insertion within their domains has been proved to be responsible for many mammary tumors in C3H and BR6 mice.[18, 19, 21] Hence, DNA rearrangements at these loci were examined in mammary plaques and carcinomas. As shown in Fig. 5, *int-1* DNA rearrangements were found in 5 of 10 carcinomas but not in any of 10 plaques. In contrast, *int-2* DNA rearrangements occurred in 2 of 10 plaques but not in any of 9 carcinomas (data not shown).

DISCUSSION

Mammary plaques were detected during first pregnancy in half of DDD-*Mtv-2* but in none of DDD mice, indicating that the *Mtv-2* gene is responsible for development of the pathologic lesion. Most of the plaque-positive mice had only one plaque and half of the plaques were not larger than 2 mm in diameter (Table I). In contrast, 90% of GR mice developed plaques: most of them had 2 or more plaques and 70% of the plaques were larger than 2 mm in diameter under the same conditions of observation (A. Matsuzawa; unpublished observation). Thus, the DDD background appeared to be more suppressive of the expression of the *Mtv-2* gene than the GR one. When mammary plaques were implanted into cleared fat pads of virgins, most of them gave rise to normal-appearing ductal-alveolar but not tumorous outgrowths (Fig. 1) as did the TPDMT-4 line.[22] HD mammary tumors from late-pregnancy GR females developed into a network of ducts resembling normal virgin mammary glands in cleared fat pads of nonpregnant hosts.[23, 24] Histologically, these tumors were composed of ductal hyperplasia and were thus considered to be equivalent to

plaques. This suggests that both plaques and HD tumors show preneoplastic properties at the hormone levels of virgins. In support of this, their responses to E or P alone were very similar to those observed in normal mammary glands.[25] In contrast, both preneoplastic tissues were different from the normal mammary tissue in the response to pregnancy and E plus P treatment.[26] Half of the plaque tissue implants in intact fat pads produced tumorous outgrowths when their hosts were pregnant. The low tumor-forming rate may be explained by the growth-inhibitory effect of normal mammary tissue as observed in HD tumors of GR mice.[24] In addition, some plaques gave rise to ductal hyperplasia in the presence of an E plus P pellet. Mammary plaques were equivalent to or more advanced than host mammary glands in degree of alveolar development, suggesting that the preneoplastic cells may be more sensitive to hormones than normal mammary epithelial cells. However, GR HD tumors appeared to be equally or less hormone sensitive in alveolar formation as compared with normal mammary glands.[23] Such a subtle difference may also be one of the background effects.

Actin characteristic of myoepithelial cells was not seen in mammary plaques from DDD-*Mtv-2*, as in those from GR mice.[27] Basement membranes visualized by anti-type IV collagen serum were well preserved in both plaques and carcinomas (Fig. 3C). Discontinuity of basement membranes is extremely rare in mouse mammary tumors.[28,29] MMTV (gp52 and p27) expression was lost in plaques, probably due to poor secretory activity.[27]

It is well established that MMTV lacking an oncogene induces mammary tumors by insertion mutation of host genes.[18-20] All the mammary plaques and carcinomas from DDD-*Mtv- 2* mice had one or more acquired MMTV proviruses (Fig. 4), clearly demonstrating that MMTV proviral insertion is prerequisite for transformation of normal to preneoplastic states, which is an early step of mammary tumorigenesis, and additionally that the *Mtv-2* gene induces mammary tumors not directly but indirectly through virus production. MMTV tumorigenesis has been characterized by a high frequency of proviral activation of the cellullar oncogenes, *int-1* and *int-2*. Thus, their rearrangements were examined in plaques and carcinomas from DDD-*Mtv-2* mice. Interestingly, *int-1* DNA rearrangement occurred only in carcinomas and *int-2* DNA rearrangement only in plaques. In the GR mouse, the donor of *Mtv-2*, rearrangement and/or activation of the *int-2* gene were detected in HD but not in hormone-independent tumors whereas those of *int-1* occurred in both HD and hormone-independent tumors.[30] These results suggest that *int-2* may have a primary role in preneoplastic transformation and *int-1* may contribute to progression to more malignant states in the *Mtv-2*-related mammary tumorigenesis.

Taken together, the results presented here indicate that mammary plaques of the new congenic strain, DDD-*Mtv-2*, will provide a useful material for research into the hormone-dependent preneoplastic state in mammary tumorigenesis.

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