The Different Activation of *int* Genes in Mammary Carcinomas Developed in Three Mouse Strains Harboring Mouse Mammary Tumor Viruses Derived from DD/Tbr

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RNA expressions of common integration site (*int*) genes and several *c*ogenes were investigated in mammary carcinomas spontaneously developed in different three strains of mice; DD/Tbr, NIH Swiss and BALB/c which harbor DD-MMTV derived from DD/Tbr mouse. Latter two strains of mice were designated NIH/Mtv⁺ and BALB/Mtv⁺, respectively. An increased expression of *int*-1 (*wnt*-1) and *int*-2 genes was observed in 56% (9/16) and 50% (8/16) of mammary carcinomas of DD/Tbr mice, respectively. Either *int*-1 or *int*-2 RNAs were expressed in 81% (13/16) of the carcinomas of DD/Tbr mice. IN NIH/Mtv⁺ mice, activation of *int*-1 and *int*-2 was observed in 41% (7/17) and 24% (4/17) of mammary carcinomas, respectively. Either *int*-1 or *int*-2 RNAs were expressed in 47% (8/17) of the carcinomas examined in this strain. In BALB/Mtv⁺ mice, on the other hand, either *int*-1 or *int*-2 genes were transcribed into RNAs at low frequency (33% : 3/9). These results suggest that the frequency of activation of *int* genes in mammary carcinomas induced by the same DD-MMTV in three strains of mice is genetically defined characteristics of these strains, and that the involvement of *int*-1 and *int*-2 genes in virus-induced mammary carcinogenesis may be influenced by genetic properties of animals. The activation of *int*-1 and *int*-2 genes did not clearly correlate with an increase in the expression of oncogenes examined; H-*ras*, K-*ras*, N-*ras*, *myc*, *raf*, *fgr*, *fms*, *erbB*, *mos*, and *src* genes. — KEY WORDS: *int* gene, mammary carcinoma, mouse mammary tumor virus, oncogene, RNA expression

Mammary carcinomas (MC) induced by mouse mammary tumor viruses (MMTV) acquire various numbers of newly integrated MMTV proviruses in addition to edogenous MMTV [10]. Some of these additional MMTV proviruses are integrated at common sites in cellular DNA of MC. The cellular DNA sequences of the common sites are molecularly cloned and designated as *int*-1 [11] (later, *wnt*-1), *int*-2 [2, 15] and *int*-3 [5]. The *int*-1 gene products are glycoproteins and seem to be

some growth and differentiation factors [1, 14]. Transcriptional activation of *int*-1 gene is an initiating event in multistep carcinogenesis [21]. Amino acid sequence of the *int*-2 protein have homology to fibroblast growth factor [3, 4, 9]. The *int*-2 gene plays multiple roles in the embryo stages of development [22]. Such *int* genes are considered to play an important role in viral mammary tumorigenesis [12]. The frequency of activation of these genes, however, varies greatly among mouse strains

[7, 12]. These facts raise the question which of MMTV and host cells determine the extent of contribution of *int* genes in viral mammary tumorigenesis.

We have previously examined DNA rearrangements of *int-1* and *int-2* regions of MCs developed in DD/Tbr, and in the progeny of NIH Swiss (NIH/Mtv⁺) and BALB/c mice (BALB/Mtv⁺), which were infected with MMTV (DD-MMTV) derived from DD/Tbr mice. In 18 MCs of DD/Tbr mice examined, the rearrangements of *int-1* and *int-2* regions were observed in 10 MCs (56%) and 2 MCs (11%), respectively. In contrast, the rearrangements of *int-1* and/or *int-2* regions were in only one (11%) out of 9 MCs in NIH/Mtv⁺ mice and only one (13%) out of 8 MCs in BALB/Mtv⁺ mice, respectively [unpublished data].

In the present study, the level of RNA expression of *int* genes were examined in MCs the three strains of mice, which were persistently infected with the DD-MMTV. Increased expressions of either *int-1* or *int-2* genes were 81% in DD/Tbr, 47% in NIH/Mtv⁺ and 33% BALB/Mtv⁺ mice. This suggests that the frequency of expression of *int* genes in mammary carcinomas depends largely on genetic background of hosts.

Materials and Methods

Mice : The origin of DD/Tbr mice was described previously [6]. Newborns of NIH Swiss and BALB/c mice were intraperitoneally injected with DD-MMTV produced by a mammary tumor cell line within 48 hours after birth [8]. These NIH Swiss and BALB/c mice were designated NIH/Mtv⁺ and BALB/Mtv⁺, respectively. While about a half of BALB/Mtv⁺ females develop MC by 20 months old, all NIH/Mtv⁺ females develop MC within 1 year after birth.

All animals were kept in air-conditioned rooms where the temperature was $24 \pm 0.5^\circ\text{C}$, the relative humidity $55 \pm 5\%$, and automatic lighting during 7:00 a. m. – 7:00 p. m.. The mice were given a commercial diet (MF, Oriental Yeast, Co.) and water *ad libitum*.

RNA extraction : RNA was extracted by the method as described previously [13].

Hybridization procedure : Hybridization probes included the following DNA fragments: MMTV LTR [11], MMTV*gag-pol* [2], MMTV*env*, *int-*

1c [11], *int-2c* and *int-2e* [2], *int-3* [5], K-*ras*, H-*ras*, N-*ras*, c-*myc*, v-*raf*, v-*fgr*, v-*fms*, v-*erbB*, v-*mos*, v-*src* and actin. Latter 8 oncogene DNAs were gifts from Japanese Cancer Research Resources Bank (JCRB)-Gene. All probes were labelled with ³²P-dCTP (3,000 Ci/mmol, Amersham) by nick translation at specific activities higher than 10^8 cpm/ μg [18]. Dot-blotted RNAs on the nitrocellulose filters were hybridized with ³²P-labelled DNA probes at 42°C for 18 hr or more, as described by Thomas [20]. X-ray films were exposed to the filters in -80°C . Relative quantities of hybridizable RNA to DNA probes were determined by a densitometer type P-2 (Fuji Film Co.). Optical densities of spots hybridized with ³²P-labelled actin were used as standard.

Results

Expression of MMTV proviral genomes : RNA expressions of MMTV in MCs from DD/Tbr, NIH/Mtv⁺ and BALB/Mtv⁺ mice were determined by the dot blot hybridization analysis using DNA probes of MMTV*gag-pol* and MMTV*env*. The expression level of RNAs hybridizable to the MMTV*gag-pol* (Fig. 1A, 1B) and MMTV*env* (data not shown) in all tumors from DD/Tbr, NIH/Mtv⁺ and BALB/Mtv⁺ mice examined were remarkably increased in comparison with those in livers from the tumor-bearing mice. All the tumors contain one or more newly acquired MMTV proviruses in addition to endogenous MMTV proviruses [unpublished data]. Increased level of MMTV transcripts may be due to newly acquired MMTV proviruses.

Activation of *int-1*, *int-2*, and *int-3* genes : The expression of *int-1* gene was examined in MCs from DD/Tbr, NIH/Mtv⁺ and BALB/Mtv⁺ mice (Fig. 2A, 2B and Table 1). Elevated expression of the *int-1* gene was observed in 9 of 16 MCs (56%) from DD/Tbr, 7 of 17 MCs (41%) from NIH/Mtv⁺, and 2 of 9 MCs (22%) from BALB/Mtv⁺ mice. Frequency of elevated transcription of *int-1* gene in MC of DD/Tbr was significantly higher than that in MC of BALB/Mtv⁺. The high level of RNA expression of *int-1* was observed in some MCs; E 50 and C 58 of DD/Tbr and M 35 and M 38 of NIH/Mtv⁺. The medium level of expression was detected in MCs of DD/Tbr (M 36, B 23, B 25 and C 61), NIH/Mtv⁺ (MT 1)

Fig. 1. Expression of MMTV proviruses in MCs from DD/Tbr (A), NIH/Mtv⁺ and BALB/Mtv⁺ mice (B). Three-fold serially diluted RNA was transferred to nitrocellulose filters at the density of 0.11, 0.33, 1.0, and 3 μ g per each spot from the top to the bottom on the same column, respectively. Dot-blotted RNA on the filters were hybridized with ³²P-labelled MMTVgag-pol probe DNA. (A) column 1: liver; columns 2 to 17: MC. (B) column 1: liver of NIH/Mtv⁺; columns 2 to 6: MCs of NIH/Mtv⁺; column 7: liver of BALB/Mtv⁺; columns 8 to 16: MCs of BALB/Mtv⁺.

Fig. 2. Activation of a putative mammary oncogene, *int-1*, in MCs from DD/Tbr (A), NIH/Mtv⁺ and BALB/Mtv⁺ mice (B). Procedures is the same to the legend to Figure 1 except with *int-1c* probe. (A) column 1: liver; columns 2 to 17: MC. (B) column 1: liver of NIH/Mtv⁺; columns 2 to 6: MCs of NIH/Mtv⁺; column 7: liver of BALB/Mtv⁺; columns 8 to 16: MCs of BALB/Mtv⁺.

Table 1. Expression of RNA in mammary carcinomas and livers of DD/Tbr, NIH/Mtv⁺ and BALB/Mtv⁺ mice

RNA sources		RNA no.	Expression of RNA hybridizable to ¹⁾				
			<i>int</i> -1	<i>int</i> -2	<i>int</i> -3	H- <i>ras</i>	K- <i>ras</i>
DD/Tbr	Lv ²⁾	DL1	—	—	—	+	+
DD/Tbr	MC ²⁾	E47	—	—	—	+	+++
DD/Tbr	MC	E49	—	—	—	++	++
DD/Tbr	MC	E50	+++	—	—	+	++
DD/Tbr	MC	B13	+	—	—	+++	ND
DD/Tbr	MC	M36	++	++	—	++	++
DD/Tbr	MC	A15	—	—	—	++	+++
DD/Tbr	MC	A16	+	—	—	+++	++
DD/Tbr	MC	B23	++	+	—	+	++
DD/Tbr	MC	B24	—	++++	+	++	+
DD/Tbr	MC	B25	++	++	—	+	++
DD/Tbr	MC	B14	—	+++	—	++	+
DD/Tbr	MC	C58	++++	—	—	+++	++
DD/Tbr	MC	C59	—	+	—	+++	ND
DD/Tbr	MC	C62	+	—	—	+++	++
DD/Tbr	MC	C60	—	++++	—	++	++
DD/Tbr	MC	C61	++	+	—	+++	+++
NIH/Mtv ⁺	Lv	NL1	—	—	—	—	—
NIH/Mtv ⁺	MC	M32	—	—	—	++	++
NIH/Mtv ⁺	MC	M33	—	—	—	++	+
NIH/Mtv ⁺	MC	M34	—	—	—	++	+
NIH/Mtv ⁺	MC	M35	++++	++	—	+	+
NIH/Mtv ⁺	MC	M38	+++	—	—	+	++
NIH/Mtv ⁺	MC	MT1	++	—	—	+	+
NIH/Mtv ⁺	MC	MT2	+	+	—	+	—
NIH/Mtv ⁺	MC	N7R	+	+	—	+	—
NIH/Mtv ⁺	MC	N8R	—	—	—	+	—
NIH/Mtv ⁺	MC	N9R	—	++	—	++	+
NIH/Mtv ⁺	MC	N10R	—	—	—	+	+
NIH/Mtv ⁺	MC	N11R	—	—	—	+	+
NIH/Mtv ⁺	MC	N12R	—	—	—	—	+
NIH/Mtv ⁺	MC	N13R	+	—	—	—	+
NIH/Mtv ⁺	MC	N15R	—	—	—	—	++
NIH/Mtv ⁺	MC	N16R	+	—	—	+	+
NIH/Mtv ⁺	MC	N17R	—	—	—	+	++
NIH/Mtv ⁺	Lv	N18R	—	—	—	+	+
BALB/Mtv ⁺	Lv	BL1	—	—	ND	—	+
BALB/Mtv ⁺	MC	A31	++	—	ND	—	+
BALB/Mtv ⁺	MC	B1	—	++	ND	+	+
BALB/Mtv ⁺	MC	B2	—	—	ND	++	+
BALB/Mtv ⁺	MC	B3	—	—	ND	—	++
BALB/Mtv ⁺	MC	B4	—	—	ND	+	+
BALB/Mtv ⁺	MC	E3	+	—	ND	—	++
BALB/Mtv ⁺	MC	E5	—	—	ND	+	+++
BALB/Mtv ⁺	MC	E9	—	—	ND	—	++
BALB/Mtv ⁺	MC	E10	—	—	ND	+	++

RNA extraction and hybridization procedure are described in Materials and Methods.

1) Relative quantities of hybridizable RNA to DNA probes were determined by a densitometer. ND : not determined. 2) Lv : liver, MC : mammary carcinoma.

Fig. 3. Activation of another putative mammary oncogene, *int-2*, in MCs from DD/Tbr (A), NIH/Mtv⁺ and BALB/Mtv⁺ mice (B). Procedures is the same to the legend to Figure 1. (A) column 1: liver; columns 2 to 17: MC. (B) column 1: liver of NIH/Mtv⁺; columns 2 to 6: MCs of NIH/Mtv⁺; column 7: liver of BALB/Mtv⁺; columns 8: to 16: MCs of BALB/Mtv⁺.

and BALB/Mtv⁺ (A 31), and the low level of expression in DD/Tbr MCs (B 13, A 16 and C 62), NIH/Mtv⁺ MCs (MT 2, N 7 R, N13R and N16R) and BALB/Mtv⁺ MC (E 3).

The level of RNA expression of *int-2* gene was also determined in RNAs from MCs and livers by dot blot hybridization analysis (Fig. 3 A, 3 B and Table 1). Its elevated level of expression was observed in 8 of 16 MCs (50%) from DD/Tbr, 4 of 17 MCs (24%) from NIH/Mtv⁺, 1 of 9 MCs (11%) from BALB/Mtv⁺ mice (Table 1). The *int-2* gene was also activated at significantly higher frequency in DD/Tbr than in BALB/Mtv⁺. In some MCs from DD/Tbr (B 24, B 14 and C 60), NIH/Mtv⁺ (M 35) and BALB/Mtv⁺ (B 1), the levels of expressed *int-2* gene were elevated (Fig. 3). Frequency of elevated activation in either *int-1* or *int-2* genes in MCs from DD/Tbr (13/16; 81%) was significantly higher than those of NIH/Mtv⁺ (8/17; 47%) and BALB/Mtv⁺ (3/9; 33%) (Table 1).

RNA transcripts of *int-3* gene was detected in only one MC (B 24) out of 16 MCs from DD/Tbr and none of 17 MCs from NIH/Mtv⁺. In all livers from three mouse strains, it was expressed at a very low or an undetectable level.

Status of expression in oncogenes: Expression

of oncogenes, *H-ras*, *K-ras*, *N-ras*, *c-myc*, *raf*, *fgr*, *fms*, *erbB*, *mos*, *src* genes, was also examined in the MCs. Although the expression of *H-ras* in livers from three strains was undetectable or low, that or the *H-ras* in most MCs from DD/Tbr mice and many MCs from NIH/Mtv⁺ and BALB/Mtv⁺ mice was elevated (Table 1). The pattern in *K-ras* expression was similar to that of *H-ras* in all strains, but the level of *K-ras* expression varied largely among individual mice (Table 1). Remarkable increase in *myc* expression was observed in almost all MCs from three strains (data not shown). Expression of *fgr* gene was also considerably elevated in most MCs of all strains when compared with livers. No obvious activation of *N-ras*, *raf*, *fms*, *erbB*, *mos* and *src* genes were observed, although RNA transcripts hybridizable with *raf*, *fms* and *mos* probes were detected in a few MCs from BALB/Mtv⁺ (data not shown).

Discussion

All mammary tumors examined contained one or more newly acquired MMTV proviruses in addition to endogenous MMTV proviruses [unpublished data]. Increased expression of

MMTV transcripts might be due to newly acquired MMTV proviruses. Some of these extra MMTV proviruses were integrated into common integration sites, *int*-1, *int*-2 or *int*-3 domains. The frequency of integration of MMTV provirus into these sites of MCs varies strikingly among mouse strains [12]. Here we showed that the frequency of MCs with increased level of expression of *int*-1 or *int*-2 gene were remarkably different among DD/Tbr, NIH/Mtv⁺ and BALB/Mtv⁺ mice. This indicates that RNA expression of *int* genes is attributable at least to genetic characteristics of host animals, because all three strains of mice harbor DD-MMTV of same origin. Thus, the genetic property of host animals is one of important determinants on the extent of *int* gene expression. MMTV-harboring inbred mouse strains, which have a high mammary tumor incidence such as C3H/He, GR, RIII, DD/Tbr and SHN, have a distinct frequency of integration for the *int* regions of host animals [7, 12]. However, when the same strain of mice are infected with MMTVs of different origins, whether these viruses have distinct preferences for *int* sites remains unknown.

Peter *et al.* (1986) reported a concerted activation of two potential proto-oncogenes in MCs from BR 6 mouse strain [17]. We also observed the concerted activation of *int* regions in DD-MMTV-induced MCs. In this study, it is considered that the same cell population would express both the *int*-1 and *int*-2 genes, because the mammary tumors might consist of a single tumor cell clone [19]. Four out of 9 *int*-1-positive of DD/Tbr were positive for *int*-2 expression, and other 5 MCs were *int*-2 negative. In NIH/Mtv⁺ mice, 3 out of 7 *int*-1-positive MCs were also *int*-2 positive, and the rest 4 were negative. Thus, the expression of *int*-1 and *int*-2 genes seemed to be regulated independently.

In transgenic mice harboring *int*-1 gene, the activation of the transgene is associated with hyperplasia of mammary gland. This means that the activation of *int*-1 gene may be associated with an initiating event in multistep carcinogenesis [24]. Proviral activation of *int*-2 is considered to be also an early event in the carcinogenesis [16], and even the tumors with activated *int*-2 gene need further additional events to become hormone independent. On the other hand, malignant activation of *H-ras*-1 oncogene was found to be involved in

the initiation process in mammary carcinogenesis by chemical carcinogen such as N-nitroso-N-methylurea [23]. In this study, expression of *int* genes did not show any correlation with that of other oncogenes as far as examined. What roles these oncogenes played in *int*-1 or *int*-2-positive MC and *int*-negative MC is not unknown. Interaction of many genes such as oncogenes and suppressor oncogenes would require to be analyzed to fully elucidate the mammary carcinogenesis.

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DD/Tbr マウス乳癌ウイルスを保持する 3 系統の

マウスの乳癌における *int* 遺伝子の発現

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DD/Tbr マウス, DD/Tbr マウス乳癌ウイルス (DD-MMTV) 感染 NIH Swiss マウス (NIH/Mtv⁺), および DD-MMTV 感染 BALB/c マウス (BALB/Mtv⁺) の乳癌における MMTV の共通組み込み部位, *int* 遺伝子の発現を調べた。DD/Tbr マウスの乳癌 16 例のうち 9 例 (56%) と 8 例 (50%) において, それぞれ *int-1* および *int-2* 遺伝子の発現が増加していた。また, この DD/Tbr マウスに発生した 16 例の乳癌のうち 13 例 (81%) において *int-1* と *int-2* のいずれかの遺伝子発現の増加がみられた。一方, NIH/Mtv⁺ マウスでは 17 例の

乳癌中 7 例 (41%), 4 例 (24%) において, それぞれ *int-1* および *int-2* 遺伝子の発現が増加していた。*int-1* と *int-2* のいずれかの遺伝子の発現増加が見られたのは, DD/Tbr に比べて明らかに少なく, 17 例中 8 例 (47%) であった。BALB/Mtv⁺ マウスの乳癌ではさらに低く, *int-1* と *int-2* のいずれかの遺伝子の発現増加を示したのは, 9 例中 3 例 (33%) に過ぎなかった。DD-MMTV で 3 系統のマウスにひきおこした乳癌における *int-1* あるいは *int-2* 遺伝子の発現増加の頻度の違いは, マウスの系統に固有の遺伝的性質によると考えられる。従っ

て、乳癌発生における *int-1* と *int-2* の寄与の大きさは宿主細胞側の遺伝的性質によって大きく変わることを示唆している。また、*int-1* および *int-2* 遺伝子の活性化は

H-ras, *K-ras*, *N-ras*, *myc*, *raf*, *fgr*, *fms*, *erbB*, *mos*, *src* のような癌遺伝子の発現と相関していなかった。