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Cancer Letters 193 (2003) 49–55
CANCER Letters
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Number of pregnancies and ovariectomy modify mammary carcinoma development in transgenic HER-2/neu female mice

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Received 30 October 2002; received in revised form 8 December 2002; accepted 10 December 2002

Abstract

We studied effect of pregnancy and ovariectomy on the development of mammary tumors in homozygous female *HER-2/neu*transgenic mice. The mean life span of uniparous mice was decreased by 16% in comparison to the control ($P < 0.05$) and of mice which have two pregnancies decreased by 11% ($P < 0.05$). Ovariectomy at the age of 2 months was followed by 32.7% increase in mean life span of mice. The incidence or multiplicity of mammary adenocarcinomas did not change in uniparous mice, whereas the size of the tumors and metastatic potential were decreased as compared to the virgins. When mice have two full-time pregnancies, there was an increase in multiplicity of mammary carcinomas and significant (2.1-fold) decrease in the survival time of tumor-bearing mice. Ovariectomy significantly decreased the total incidence of mammary carcinomas, the number of tumors per tumor-bearing animal, and inhibited metastasizing into lungs. Our results indicate that pregnancy accelerated the development of mammary adenocarcinomas in transgenic *HER-2/neu* mice whereas ovariectomy inhibits their development.

Keywords: *HER-2/neu* transgenic mice; Pregnancy; Ovariectomy; Mammary carcinoma

1. Introduction

Breast cancer is one of the most common cancers, and a leading cause of mortality, in women [1,2]. Several studies have demonstrated that a full-term pregnancy exerts a short-term adverse influence and long-term beneficial influence on breast cancer risk [3–6]. Comparing uniparous with nulliparous women the transient increase in maternal breast cancer risk peaked 5 years following delivery (odd ratio = 1.49), and biparous women had a transient increase in the risk that was lower at its peak than that of uniparous women [7]. In mice, spontaneous mammary tumors occurred in 4% of the virgin females of substrain C3HeB at an average age of 20.7 months, in 54.8% of breeding females at the average age of 19.2 months, and in 74.4% of force-bred females at an average age of 17.9 months [8].

The *HER-2/neu* oncogene encodes a 185 kDa (p 185) receptor protein belonging to the epidermal

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0304-3835/03/$ - see front matter © 2003 Elsevier Science Ireland Ltd. All rights reserved.
doi:10.1016/S0304-3835(02)00721-8

growth factor receptor family involved in organogenesis and epithelial differentiation [9]. Amplification and mutation of *HER-2/neu* plays a pathogenetic role in several malignancies, including carcinoma of the breast, ovary and uterus [10]. Overexpression of *ErbB-2/HER-2/neu* occur in 15–40% of human breast cancers and its appearance is correlated with poor prognosis and is, therefore, an important target for physiologic investigation and therapeutic intervention [10–12]. In contrast to the slightly protective effect of full pregnancy and of breast-feeding in the *HER-2/neu*-negative women, the risk of *HER-2/neu*-positive breast cancer was 4.2-fold increased in women who ever breast fed [13]. Transgenic mice represent useful modes for assessing the role of oncogenes in vivo. Transgenic mice were generated that express the activated *HER-2/neu*oncogene under the transcriptional control of a mammary-gland-specific promoter. This promoter is contained in the long terminal repeat of the mouse mammary-tumor virus (MMTV-LTR) [14,15]. In this paper the data presented on effect of number of full-term pregnancies and ovariectomy on development of mammary tumors in transgenic *HER-2/neu* mice.

2. Material and methods

2.1. Animals

Homozygous *HER-2/neu*transgenic mice obtained from Charles River (Hollister, CA) by the Italian National Research Center for Aging were housed and breed in the Laboratory of Carcinogenesis and Aging. The mice were kept 5–7 in polypropylene cages ($30 \times 21 \times 10$ cm) under standard light/dark regimen (12/12 h) at temperature 22 ± 2 °C and received standard laboratory chow [16] and tap water ad libitum.

2.2. Experimental design

One hundred and one female FVB/N *HER-2/neu* mice at the age of 2 months were randomly divided into four groups. Mice from group 1 ($n = 30$) were virgin and served as controls. Forty six mice from groups 2 and 3 were mated with males, became pregnant, have a delivery and kept with pups during one month. Then they were again mated with males. Some of them had one more offspring and some not. Those mice which have only one parity were included into the group 2 ($n = 21$) and those which have two parities were included into the group 3 ($n = 25$). Twenty five virgin females were surgically ovariectomized at the age of 2 months and were included into the group 4.

Once a week all mice were palpated to detect mammary tumors. Localization and size of tumors were registered on special charts. The time of appearance of mammary tumors was evaluated by palpation and neoplastic masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Because some treated mice did not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas/-mouse was calculated as the cumulative number of incident tumors/number of tumor-bearing mice. Animals were observed until natural death. The date of each death was registered, and mean life span, the age by which 90% of the animals died, and maximal life span estimated.

2.3. Pathomorphological examination

All animals were autopsied. Site, number and size of mammary tumors and metastases to lungs were checked. All tumors, as well as tissues and organs with suspected tumor development, were excised and fixed in 10% neutral formalin. After fixation the number of metastases in each lung lobe as well as size of metastases, were estimated as recommended by the International Agency for Research on Cancer [17]. After routine histological processing, tissues were embedded in paraffin. 5–7 μm thin histological sections were stained with haematoxylin and eosin and were microscopically examined. Tumors were classified according to the International Agency for Research on Cancer recommendations [17] and Annapolis Consensus Report [18].

2.4. Statistics

Experimental results were statistically processed using STATGRAPH. The significance of discrepancies was defined according to the Student's *t*-criterion, Fischer's exact method, χ^2, For survival analysis, Cox's method [19] was used for testing two groups. All reported test values for survival analyses are two sided.

2.5. Mathematical models and estimations

The mathematical model used to describe survival is the Gompertz model with the survival function:

$$S(x) = \exp\left\{-\frac{\beta}{\alpha}[\exp(\alpha x) - 1]\right\}$$

where parameters α and β are associated with demographic aging, and initial mortality rate, respectively. Parameters for the model were estimated from data using the maximum likelihood method implemented in the GAUSS statistical system [20].

3. Results

The data on the dynamics of survival of mice in different groups are presented in Fig.1.

One can see from Fig. 1 that the survival of parous mice was decreased as compared to the virgins, whereas it was significantly increased in ovariectomized animals. Survival curves of both uniparous and biparous mice were shifted to left as compared to that in the virgins, and it was significantly decreased in mice subjected to ovariectomy at the age of 2 months. The mean life span of uniparous mice was decreased by 16% in comparison to the control ($P < 0.05$) and of biparous mice decreased by 11% ($P < 0.05$). Ovariectomy was followed by 32.7% increase in mean life span of mice. The pregnancies also decreased the mean life span of last 10% of survivors. One pregnancy resulted in slowing down the demographic ageing rate in comparison to the virgins, whereas two pregnancies increased it (Table 1). A single parity did not increase mammary adenocarcinoma's incidence or multiplicity of the malignancies in mice (Fig. 2), decreased their size and metastatic potential. However, survival of mice since the first tumor detection was strictly reduced in this group. When mice have two pregnancies, there was an increase in multiplicity of mammary carcinomas and significant (2.1-fold) decrease in the survival time of tumor-bearing mice after the first tumor detection. Ovariectomy significantly decreased the total incidence of mammary carcinomas, the number of tumors per tumor-bearing animal, and inhibited metastasizing into lungs (Table 2). The relative number of mice bearing five and more mammary tumors was proportional to a number of parity, whereas in ovariectomized mice its number was minimal (Fig. 3). At the microscopic examination all tumors of a mammary gland were classified as adenocarcinomas type B [18] and revealed a solid, lobular and cribrosis structure with multiple hemorrhagic cysts. There was no difference in the morphological structure of mammary carcinomas between all groups.

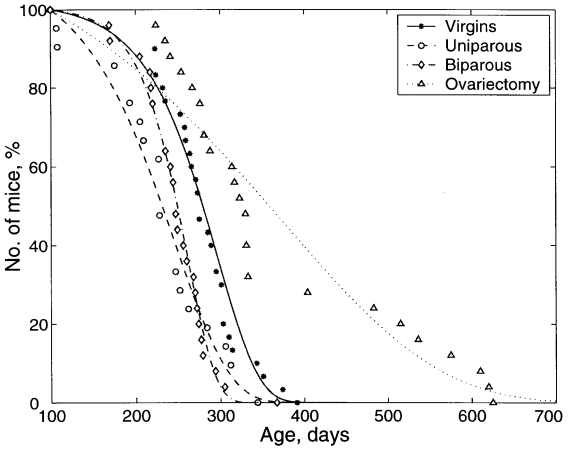

Fig. 1. Survival curves of virgin, parous and ovariectomized female *HER-2/neu* transgenic mice. Pregnancy (one or two) reduces survival whereas ovariectomy increases it in the transgenic mice.

4. Discussion

The full-term pregnancy is an important factor of

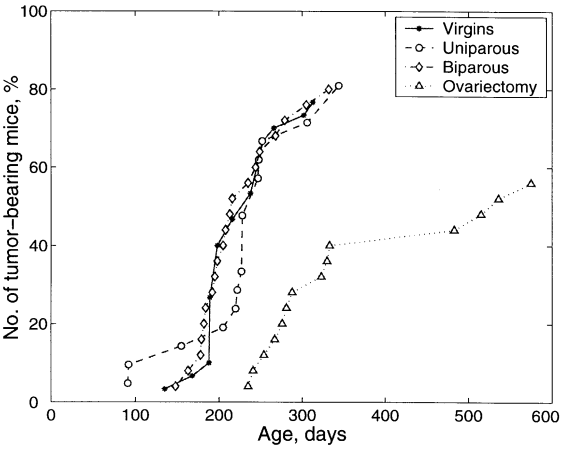

Fig. 2. Mammary adenocarcinoma yield curves in virgin, parous and ovariectomized female *HER-2/neu* transgenic mice. Pregnancy (one or two) failed influence total mammary tumor incidence in *HER-2/neu* transgenic mice whereas ovariectomy significantly decreases the mammary tumor incidence and increases the latency of them.

Table 1
Effect of a number of deliveries and ovariectomy on parameters of life span in HER-2/neu transgenic mice[a]

Parameters	Virgin	Uniparous	Biparous	Ovariectomy
Number of mice	30	21	25	25
Mean life span, days (M ± S.E.)	281 ± 8.1	236 ± 13.8*	250 ± 8.3*	373 ± 26.4**
Median	275	228	247	330
Mean life span of last 10% of survivors	372 ± 11.6	344 ± 0	367 ± 22.7	618 ± 4.4*
Maximum life span	391	344	367	625
$\alpha \times 10^2$ (day^{-1})	2.31 (2.08; 2.54)	1.72 (1.58; 1.86)#	3.13 (2.84; 3.41)#	0.59 (0.55; 0.64)#
MRDT, days	29.97	40.28#	22.18#	168.18#

[a] Mean life spans are given as mean ± standard error; 95% confidence limits are given in parentheses; MRDT = mortality rate doubling time. The difference with the virgins is significant, *$P < 0.01$; **$P < 0.05$ (Student's t test); #$P < 0.05$ (Cox's method).

risk of breast carcinoma in women. It was shown that risk of breast cancer increased in the first 5 years after pregnancy in comparison with nulliparous women, however biparous women revealed lower risk than uniparous ones [7]. The genetic factor is extremely important for breast cancer initiation and development and, as a whole in the prognosis of future status in cancer patients. Overexpression of *HER-2/neu*oncogene observed in a 15–40% of breast cancer and correlated with poor prognosis [21]. In *HER-2/neu*-negative women, pregnancy followed by breast feeding has slightly protective effect, however the risk of breast cancer significantly increases after pregnancy in *HER-2/neu*-positive women [13]. Our data are in agreement with these observations. Thus, survival of uniparous and biparous transgenic *HER-2/neu* mice was significantly reduced in comparison to the virgins. The relative number of mice bearing five and more mammary carcinomas was increased in both groups of parous mice as compared with virgins (Fig. 3). Breeding and pregnancy increased the incidence of mammary carcinomas in mice of various strains [8,22,23]. It was shown that parity results in a persistent increase in the differentiated state of mammary gland. It also yields persistent down-regulation of multiple genes encoding growth factors, such as amphiregulin, pleiotrophin, and insulin growth factor-1, as well as the persistent up-regulation of the growth-inhibitory molecule, transforming growth factor-β3, and several of its transcriptional targets [24]. Using substrain of transgenic *HER-2/neu* mice developing tumors after repeated pregnancies and lactation periods, Lazar et al. [25] have shown that tumor cells over-expressing *HER-2/neu* fail to terminally differentiate at the end of

Table 2
Effect of a number of deliveries and ovariectomy on mammary adenocarcinoma (MAC) development in HER-2/neu transgenic mice[a]

Parameters	Virgin	Uniparous	Biparous	Ovariectomy
Number of mice	30	21	25	25
Number of MAC-bearing mice	23 (76.7%)	17 (81.0%)	20 (80.0%)	14 (56.0%)*
Time of the first MAC detection, days	135	91	148	127
Mean latency of the first MAC, days	210 ± 9.9	229 ± 16.9	219 ± 10.8	246 ± 31.6
Total number of MAC	75	65	83	38
Number of MAC per tumor-bearing mouse	3.3 ± 0.20	3.8 ± 0.44	4.2 ± 0.54	2.7 ± 0,30
Maximal tumor size, cm	1.88 ± 0.15	1.28 ± 0.11*	1.71 ± 0.12	1.38 ± 1.1
No. of mice with lung metastases of MAC	10 (33.3%)	1 (4.8%)**	7 (28.0%)	3 (12%)*
Maximal metastasis size, cm	0.38 ± 0.04	0.10	0.31 ± 0.05	0.65 ± 0.18
Mean survival time of tumor-bearing mice after the first MAC detection, days	76 ± 15.9	9 ± 3.4*	37 ± 7.5**	109 ± 19.9

[a] The difference with the virgins is significant, *$P < 0.01$; **$P < 0.05$.

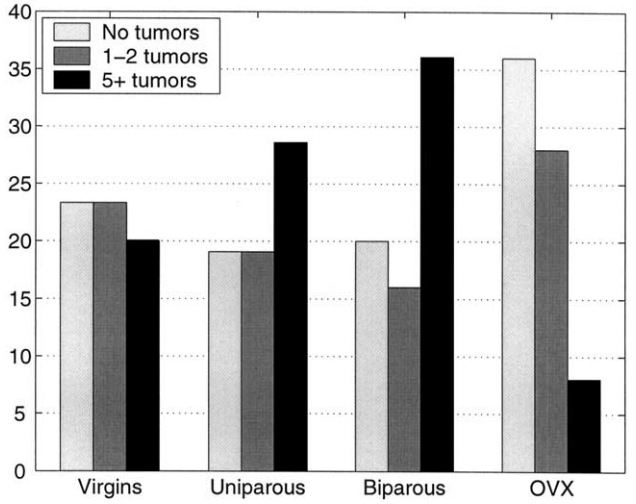

Fig. 3. Distribution of mammary adenocarcinomas in virgin, parous and ovariectomized female *HER-2/neu* transgenic mice. Pregnancy (one or two) increases the number of mice bearing five and more mammary adenocarcinomas whereas ovariectomy significantly decreased the multiplicity of mammary adenocarcinomas in the transgenic mice. Ordinate, number of mice with mammary tumors, percentage. Total number of tumor-bearing mice was regarded as 100% in each group.

pregnancy and during lactation and are mostly refractory to the developmentally controlled apoptosis during involution. Long term expression of HER-2/ne leads to abnormal lobuloalveolar development in virgin glands and incomplete regression in multiparous mammary glands [15]. It is worth noting that in uniparous mice the mean maximum tumor size was decreased in comparison with the virgins and the biparous mice (Table 2). Also the uniparous mice revealed the decrease in lung metastasis incidence as compared with both the virgins and biparous mice ($P < 0.05$). However, survival of uniparous mice was reduced as compared with the virgins and was not significantly differ from the biparous animals (Table 2; Fig. 1).

Ovariectomy at the age of 2 months dramatically reduced tumor incidence and multiplicity, decreased the incidence of lung metastases and increased the survival of transgenic *HER-2/neu* mice. In rats, early ovariectomy decreased the incidence of mammary carcinomas induced by directly inserting activated *neu* in situ rat mammary ductal cells and extended their latency [26]. Carcinomas induced by *neu* in ovariectomized rats had down-regulated estrogen receptor and progesterone receptor [26]. Preliminary estimation have shown that the level of estrogen receptors in mammary tumors in our transgenic *HER-2/neu* mice was rather low (< 5 pM/g) (E.V. Tsyrlina, personal communication). In estrogen receptor-α (ERα) knockout mice expressed MMTV-neu/erb*B2* transgene the mammary tumor onset was significantly delayed [27]. The authors suggest that ERα is not required for mammary tumor induction by overexpression of *neu/erB2*, but plays a role in the rate of tumor onset. The removal of ovarian steroids by ovariectomy in adults did not alter the onset rate, whereas prepubertal ovariectomy significantly delayed onset of mammary tumors [27]. In our experiments, the first mammary tumor in ovariectomized mice was detected at the age of 127 days, where as in the virgins at the day of 135. The mean latency of the first mammary carcinoma was also insignificantly different between these groups. However, the survival of mice ovariectomized at the age of 2 months was significantly increased, and tumor yield curve was significantly shifted to the right as compared with the virgins (Figs. 1 and 2). There is a clinical evidence of the effectiveness of prophylactic surgery for women at high risk for breast cancer [28,29]. Thus, our observations suggest at least partial dependency of *HER-2/neu*-positve mammary tumors on estrogen level and effectiveness of ovariectomy at the adult life.

Acknowledgements

This study was supported in part by grant 02-04-07573 from the Russian Foundation for Basic Research. The authors are very thankful to James W. Vaupel for the opportunity to use the facilities of the Max Planck Institute for Demographic Research to complete this paper, and to I. I. Mikhailova and T.B. Polyakova for excellent technical assistance.

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