行政院國家科學委員會專題研究計劃成果報告

台灣乳癌病理成因之研究-乳癌檢體中含變異之動情激素接受體與臨床上之關聯性(第三年)

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中文摘要

關健詞:乳癌/動情素接受體

為了研究臺灣乳癌檢體中所含變異型動情素接受體α與臨床上預後的關聯性，我們從 199 個原發性的乳癌檢體中挑選出 98 個檢體，並取其中 19 個檢體的正常對照組織，以及 8 個良性的乳腺檢體為對照組，檢查它們的密碼區(coding region)。我們在 38 個其密碼區被整個檢查過的病例中發現 1 個點突變及 3 個多形性區域，該點突變(Pro324Ser)發生於動情素接受體α的 LBD 的第一個α螺旋的末端，而 proline 一般相信對蛋白質的二級結構及局部的方向性有很大的影響。而為了進一步了瞭 3 個多形性區域的生理意義，我們將上述的其它部分檢體進行多形性區域的分析，發果發現腫瘤檢體中這 3 個多形性區域在統計分析中顯示出極度的分佈不平衡現象，而且其中有一個多形性區域顯示在癌症組織中的偏好表現，然而與癌症的復發並不具有顯著相關。而比較 19 個正常對照組織與其腫瘤檢體的多形性分佈情形，我們發現多形性區域的分佈不平衡現象可能與腫瘤組織失去同時表現兩個 alleles 的能力有關。比較腫瘤與正常組織中 exon 刪除型動情素接受體α的表現情形時，以往曾經被報告過的刪除型動情素接受體α亦都存在於我們的檢體中，我們更發現了二型從未被報告過的刪除型動情素接受體α--單獨刪除 exon 1 最後 32 個核　酸，以及此 32 核　酸連同 exon 2 一同刪除，此二型變異型均廣泛的表現於乳癌的檢體之中。此外，在乳癌檢體中所表現的各種變異型動情素接受體α之對野生型動情素接受體α相對量均較正常對照組織為高。但經由統計分析並無法測量出變異型動情素接受體α的表現與腫瘤復發之間的關聯性。

英文摘要

Key Words: Breast Cancer / Estrogen Receptor

To investigate the correlation between the presence of ERα variants and prognosis of breast tumors from Taiwan, we detailed examined the coding region of ERα gene. One sense point mutation and 3 polymorphisms have been found. The point mutation, P324S, is located at the end of the beginning helix of ER ligand binding domian. The 3 polymorphisms showed strong disequilibrium in allele distribution and one of the polymorphisms showed allele preference in tumor. However, the allele preference is not significantly associated with tumor recurrence. To elucidate the possible reason of disequilibrium in allele distribution, we compared polymorphisms of paired tumor-normal counterpart tissues, and found the disequlibrium may due to a loss expression of one allele phenomenon. In searching for exon deletion variants, alternative spliced variants were found. Moreover, a deletion of 32 nucleotide 3' to the exon 1 only (d32nt) or together with the whole exon 2 (d32nt-D2) through aberrant splicing, which has never been reported, was found to exist extensively in tumor tissues in our study. In addition, types and amount of deletion variants were more dominant in tumor tissue than normal tissues. However, the elevated expression of deleted variant

forms did not showed significant association with tumor recurrence.

Introduction

Estrogen exposure is one of the major risk factors of breast cancer development. Many epidemiological surveys support this relationship (reviewed in Pike et al. 1993). The biological mechanism is poorly understood. One hypothesis is the estrogen metabolic derivatives play a genotoxic role in the carcinogenesis of breast tissue. The other possible mechanism is the cell proliferation-promoting ability through estrogen receptor(ER) pathway. There are two known estrogen receptors-- ERa and ERβ. ERa protein plays a major role in the development and normal physiology of the breast tissue and is important for the regulation of cell growth and differentiation. To investigate the correlation between the presence of ERα variants and prognosis of breast tumors from Taiwan, we detailed examined the coding region of ERα gene from 98 out of 199 primary breast cancers, 19 normal counterparts and 8 benign masses.

Results

Detection of Nucleotide Changes by PCR-SSCP Analysis PCR-SSCP analysis was used to detect small insertions/deletions and point mutations present in ERα gene. Nine overlapped amplification regions (about 250 base pairs each in size) were carefully examined and only 1 point mutation and was found from these 38 putative ERα misfunctioning cases. The point mutation, which changed a proline residue (CCC) to serine (TCC) at codon 324, is located in the end of the beginning helix (H1, numbered based on the canonical structure of NR LBD, Wurtz et al., 1996) of the ligand binding domain. Since a proline residue may play a role in determination and orientation of local secondary structure, this mutant is possibly with altered function. However, functional assay of this mutant has not been conducted yet.

Detection of Polymorphisms by PCR-SSCP Analysis 3 neutral polymorphisms were found in the 38 putative ERα-misfunctioning cases by PCR-SSCP analysis. These polymorphiic sites are located in codon 10 [TC<u>T</u>→TC<u>C</u> (Ser), exon 1], codon 325 [CC<u>C</u>→CC<u>G</u> (Pro), exon 4], and codon 594 [AC<u>A</u>→AC<u>G</u> (Thr), exon 8], respectively. Polymorphism patterns of ERα-PR consistent and normal group were also analyzed in order to compare with those of the ERα-PR inconsistent group. When the alleleic distribution pattern of these polymorphic regions being examined, all of the 3 polymorphisms in the ERα-PR inconsistent group showed linkage disequilibriums but only polymorphism in codon 594 showed a slight allele preference (Table 1).

Detection of Exon Deletion Variants by PCR-Southern Blotting Analysis In the PCR-Southern blotting based detection of truncated transcripts, truncated transcripts (such as clone 4) or longer exon deletion variants (such as D2-3-4) can not be detected by our strategy. We could not also discriminate transcripts with single exon deletion or combine exon deletion (for instance, exon 4 and exon 7 deletions were found in the same transcript). But these transcripts present in relatively low level and the majority of variant transcripts bearing shorter deletions. In our study, the alternative spliced transcripts, D2, D3, D4, D5, D7, D2-3, D3-4, D4-5, reported by previous studies of other groups were also detected. We also found a truncated transcript comprising 32 nucleotides 3' to the exon 1 (denoted as d32nt) which has never been reported in previous publishes. It might because the shorter PCR spanning region in our study is favored finding out

small deletions. The 32 nt bears a sequence character with a beginning of GT and an ending of AG, which is seemly the feather of most introns. Thus the d32nt may owe to be mistaken as an intron and spliced out. A deletion of this 32 nt together with the whole exon 2 (denoted as d32nt-D2) was also found in our studies. Protein translated from d32nt and d32nt-D2 variants will terminated prematurely just after Thr140 in addition to 5 or 35 more extra amino acids and bearing only an incomplete A/B domain possibly without any biological activity. Compared with the basal level by quantifying the relative ratio of variant to wildtype bands, tumor groups beard significant increase in D4, D5, D7, and D2-3 expression (Table 2).

Discussion

In the present study for the search of ERα variants, we used a preliminary IHC analysis to raise the possibility of finding out ERα variants with altered function. However, we only found 1 sense point mutation out of 38 primary breast cancer cases, much fewer from the expected number, 2~10 mutations based on multiply 1~5% reported mutation rate and 199 cases. It is possible that some mutations with unaltered function may exist in the 161 cases we did not detailed screened. 2 variants comprising deletion of 32 nucleotides 3_ to exon 1 only (d32nt) or together with the whole exon 2 (d32nt-D2) were first reported in this study. They were probably generated from aberrant splicing mechanism since the 2 sequences are flanked with genuine or cryptic splice sites. Aberrant splicing mechanism is reported important in altering normal function of some cellular proteins but aberrant splicing variant of ERα was never identified except a 69-nucleotide insertion between exon 5 and 6. The variant was resulted from a new splice donor site generated by a point mutation in the intron 5 (Wang et al., 1997). However, it is the first time that aberrant splicing is found to occur within the ERα coding sequences. It is also the first time to suggest that aberrant splicing may play some role in altering the signaling pathway of ERα gene. Since these variants were only reported in Taiwan population, more extensive studies must be undertaken in different populations to determine whether they are ethnic-specific variants or they were found in the advantage of shorter PCR amplified region comparing with those used by other groups. By using polymorphisms as genetic markers, we also found significantly strong disequilibrium in allele distribution comparing with the result by an U.S. group (Roodi et al., 1995) Only 2 out of 6 polymorphisms showed relatively slighter significance. Furthermore, the polymorphisms they analyzed did not reveal the loss expression of one allele phenomenon we observed in our cases. It is thus strongly suggested that changes in ERα signaling may play a different role in breast cancer tumoriogenesis in Taiwan.

Table 1. Statistical Analysis of Differences in Allele Frequencies of ER-PR Inconsistent, ER-PR Consistent, and Benign/ Normal Counterpart Groups at the Polymorphic Regions

Allele Frequency (0/1)	Codon 10	Codon 325	Codon 594
ER-PR Inconsistent group	39/37 (51.3%/48.7%)	34/42 (44.7%/55.3%)	33/39 (45.8%/54.2%)
ER-PR Consistent group	52/32 (61.9%/38.2%)	47/43 (52.2%/47.8%)	19/59 (25.6%/74.4%)
Benign/ Normal Counterpart group	21/15 (58.3%.41.7%)	26/28 (48.1%/51.9%)	13/23 (36.1%/63.9%)
***p* value**	0.163886	0.627817	**0.022135**

Allele frequencies of the 3 groups in each polymorphic region were referred from Table 1 and

analyzed by Chi-squared analysis to tell any difference in allele distribution of those groups.

Table 2. Frequencies of Elevated Expression of ER~ Variant mRNAs in the ER~-PR Inconsistent, Consistent, and Normal Group

Putative Function of ER~ Variants	Inconsistent Group	Consistent Group	Normal Group	*p* value
No Function				
D2	20/38 (52.63%)	4/11 (36.36%)	3/14 (21.43%)	0.116668
D4	17/38 (44.73%)	5/11 (45.45%)	1/14 (7.14%)	**0.035118**
D2-3	18/38 (47.37%)	1/11 (9.09%)	1/14 (7.14%)	**0.004524**
D3-4	20/36 (55.56%)	6/11 (54.54%)	4/14 (28.57%)	0.213182
D4-5	6/37 (16.22%)	0/11 (0%)	1/14 (7.14%)	0.281277
d32nt-D2	15/38 (39.47%)	5/11 (45.45%)	1/14 (7.14%)	0.058034
Dominant Negative				
D3	18/38 (47.37%)	4/11 (36.36%)	5/14 (35.71%)	0.06299
D7	17/38 (44.74%)	6/11 (54.55%)	0/14 (0%)	**0.004738**
Constitutive Active				
D5	9/37 (24.32%)	0/11 (0%)	0/14 (0%)	**0.028528**

Whole exon deletions were abbreviated as D[exon number] and deletion of a short stretch of nucleotides was denoted as d[nt]. Chi-squared analysis was used to tell any difference in expression pattern of each variant form or functional group.

References

Brzozowski, A. M., A. C. W. Pike, Z. Dauter, R. E. Hubbard, T. Bonn, O. Engström, L. Öhman, G. L. Greene, J.-Å. Gustafsson, & M. Carlquist. (1997) Molecular Basis of Agonism and Antagonism in the Oestrogen Receptor. Nature. **389:** 753-758.

Castles, C. G., D. M. Klotz, S. A. W. Fuqua, & S. M. Hill. (1995) Coexpression of Wild-type and Variant Oestrogen Receptor mRNAs in a Panel of Human Breast Cancer Cell Lines. Brit J Cancer. **71:** 974-980.

Fuqua, S. W. A., & D. M. Wolf. (1995) Molecular Aspects of Estrogen Receptor Variants in Breast Cancer. Breast Cancer Res Treat. **35:** 233-241.

Hohaus, S., L. Funk, S. Martin, R. F. Schlenk, A. Abdallah, U. Hahn, G. Egerer, H. Goldschmidt , A. Schneewei, N. Fersis, S. Kaul, D. Wallwiener, G. Bastert, & R. Haas. (1999) Stage III and Oestrogen Receptor Negativity Are Associated with Poor Prognosis After Adjuvant High-Dose Therapy in High-Risk Breast Cancer. Brit J Cancer. **79:** 1500-1507.

Leygue, E., A. Huang, L. C. Murphy, & P. H. Watson. (1996) Prevalence of Estrogen Receptor Variant Messenger RNAs in Human Breast Cancer. Cancer Res. **56:** 4324-4327.

Murphy, L. C., H. Dotzlaw, A. Coutt, & P. Watson. (1998) The Pathophysiological Role of Estrogen Receptor Variants in Human Breast Cancer. J Steroid Biochem Molec Biol. **65:** 175-180.

Rochefort, H., N. Platet, Y. Hayashido, D. Derocq, A. Lucas, S. Cunat, & M. Garcia. (1998) Estrogen Receptor Mediated Inhibition of Cancer Cell Invasion and Motility: An Overview. J Steroid Biochem Molec Biol. **65:** 163-168.

Southey, M. C., L. E. Batten, M. R. E. McCredie, G. G. Giles, G. Dite, J. L. Hopper, & D. J. Venter. (1998) Estrogen Receptor Polymorphism at Codon 325 and Risk of Breast Cancer in Women Before Age Forty. J Natl Cancer Inst. **90:** 532-536.