Clonal evolution of human cancers

-Pathology-based microdissection and genetic analysis precisely demonstrates molecular evolution of neoplastic clones-

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The lecturer started manual microdissection technique before the advent of laser assisted microdissection system in the early 1990's. For each tumor case, multiple portions of the tumor were individually microdissected. This pathology-based microdissection or topographical microdissection and subsequent genetic analysis including loss of heterozygosity (LOH), gene mutation, microsatellite instability (MSI), methylation, gene expression analysis have revealed genetic alterations in the process of clonal tumor progression more precisely than the bulk tissue analysis. In the presentation, I will mostly introduce our previous works rather than the overview the recent molecular pathology in general.

Loss of heterozygosity (LOH): LOH is the loss of one allele at a specific chromosomal locus, caused by a deletion or loss of a chromosome/chromosomal region from a chromosome pair, resulting in hemizygosity. It is detected when heterozygous markers such as microsatellie markers for a locus appear monomorphic because one of the alleles was deleted. When this occurs at a tumor suppressor gene locus where one of the alleles is already abnormal, it can contribute to neoplastic transformation. Detection of LOH is especially usuful for the clonality analysis as shown below.

Detection of frequent allelic loss of 6q23-q25.2 in microdissected human breast cancer tissues, and potential breast cancer related gene, HIVEP2:

In our initial work by microdissection, we focused on the allelic loss analysis of the long arm of chromosome 6 (6q). We found that up to 80% to have allelic loss of either the entire chromosomal arm or a portion of the chromosomal arm. One common region that was identified for all tumors with deletions of 6q was the area between markers D6S310/314 and D6S473/255, consistent with a tumor suppressor gene locus at 6q23-6q25.2.

Subsequently, we found that the HIVEP2 gene, located on 6q23-q24, belongs to a family of genes that encodes large zinc fingers containing transcription factor proteins, are frequently downregulated in microdissected breast cancer tissue. Although this gene has been implicated in the regulation of immune responses and cellular proliferation, its functions are largely unknown. Down-regulation of the HIVEP2 genes frequently occurs and may be one of the genetic events responsible for breast cancer, and their transcription may be regulated by complex mechanisms involving interactions with other factors and/or by other genetic/epigenetic mechanisms. In the literature, other genes on 6q also reported to be related to breast cancer.

Genetic Progression, Histological Grade, and Allelic Loss in Ductal Carcinoma in situ of the Breast:

To investigate the relationships of specific allelic losses to progression and histological grade of ductal carcinoma in situ (DCIS) of the breast, we studied LOH on ten chromosomal arms in cases of DCIS without synchronous invasive cancer. For all chromosomal arms combined, the number of allelic losses was significantly greater in lesions of intermediate or high nuclear grade (5.6 chromosomal arms/case) than in lesions of low nuclear grade (1.2 chromosomal arms/case). Allelic losses of 16q and 17p were commonly found in low nuclear grade DCIS (38 and 34%, respectively) as well as in intermediate and high nuclear grade DCIS (58 and 95%, respectively). Allelic losses of other chromosomal arms examined (1p, 1q, 6q, 9p, 11p, 11q, 13q, and 17q) were uncommonly seen in low-grade DCIS, but were seen at frequencies of greater than 40% in intermediate- and high-grade DCIS. In 10 of the cases (24%), we identified patterns of allelic loss heterogeneity suggestive of intralesional progression. For these tumors with allelic loss heterogeneity, we reasoned that chromosomal losses common to all tumor foci most likely preceded the chromosomal losses observed only in tumor foci of a more advanced genetic stage. We found that chromosomal losses of 16q and 17p occur early in DCIS progression and are common even in low-grade DCIS. Tumors of intermediate and high nuclear grade usually have allelic losses of significantly more chromosomal arms, often including 1p, 1q, 6q, 9p, 11p, 11q, 13q, and 17q. Allelic loss of these chromosomal arms may occur later in DCIS progression by genetic progression.

Genetic divergence in the clonal evolution of breast cancer:

The progression of ductal carcinoma in situ (DCIS) to infiltrating and metastatic cancer

of the breast is thought to be a consequence of clonal expansions of neoplastic cells with progressively more genetic alterations. To study this progression, we dissected multiple foci from cases with synchronous DCIS and infiltrating cancer. The patterns of allelic losses identified in the in situ cancers were generally conserved in the synchronous infiltrating tumors, supporting the paradigm that the infiltrating tumors are clonally derived from the in situ lesions. However, in 8 (40%) of the 20 cases with synchronous in situ and invasive cancer, heterogeneous patterns of allelic loss at one or more chromosomal loci were observed in adjacent DCIS foci. The patters of clonal genetic progression and divergence is shown in Figure 1. We found similar patters in the pancreas cancer.

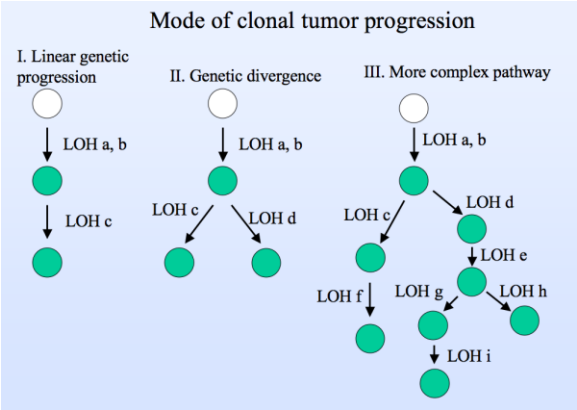

Figure 1

Genetic progression and divergence in pancreatic carcinoma:

Similar to the breast cancer evolution, we found genetic association between intraductal lesions and invasive cancer foci. For each case, allelic loss was frequently observed on 9p (severe ductal dysplasia- carcinoma in situ 90%, invasion 100%), 17p (severe ductal dysplasia or carcinoma in situ 80%, invasion 80%), and 18q (severe ductal dysplasia or carcinoma in situ 88%, invasion 88%). The patterns of allelic loss identified in severe ductal dysplasia were generally conserved in synchronous infiltrating tumors, supporting the paradigm that infiltrating tumors are clonally derived from severe ductal dysplasia-carcinoma in situ. In some cases, however, we found frequent genetic heterogeneity in the intraductal lesion, suggestive of genetic progression or divergence. These findings indicate that invasive pancreatic carcinoma evolves through successive and divergent genetic changes with selection of aggressive subclones in the intraductal component.

Comparison of loss heterozygosity in primary and recurrent ductal carcinoma in situ of the breast:

Ductal carcinoma in situ (DCIS) of the breast is often an indolent disease, although some cases are reported to recur many years after a limited surgical resection. It is not known whether these recurrences reflect a resurgence of residual disease or an independent development of a second tumor in susceptible individuals. Therefore, we conducted a longitudinal LOH study of four women with reappearance of DCIS 2 to 15 years after an initial conservative resection. In three cases with ipsilateral recurrent disease, all of the allelic losses seen in the initial tumors were also seen in the recurrent lesions, suggesting a common genetic pathway for the development of both lesions and continuous proliferation of residual disease. The presence of at least one additional LOH in all of the three recurrent tumors, however, suggests that the recurrent tumors developed after genetic progression. In contrast, in one case of DCIS that was followed by the development of DCIS in the contralateral breast 7 years later (a case of bilateral DCIS), unrelated LOH patterns were present in the two lesions. These findings suggest that the reappearance of DCIS in the same breast is most commonly the result of a tumor derived from (but not identical to) the original lesion, with acquisition of additional genetic changes, even when the recurrent lesion manifested itself many years (15 years, in one case) after the initial presentation. Furthermore, genetic progression could be detected in tumors recurring in as little as 2 years after the initial resection.

Mucinous cancers have fewer genomic alterations than more common classes of breast cancer:

Mucinous cancers of the breast are distinguished histologically by their abundant pools of mucin and low degree of nuclear pleomorphism. Relative to the more common ductal carcinomas, mucinous cancers have a relatively favorable prognosis. In a study of chromosomal changes in mucinous cancers, we evaluated the extent of LOH at markers on chromosomal arms 1p, 1q, 3p, 6q, 8p, 9p, 11p, 11q, 13q, 16q, 17p, and 17q. Remarkably, we found an average frequency of LOH of only 1.9 of these 12 chromosomal arms in 18 cases of mucinous carcinoma, compared to an average frequency of LOH of 6.4 of these same chromosomal arms in cases of infiltrating ductal cancer. In three of the 18 cases, no LOH was seen at any of the 12 chromosomal regions studied. Comparative genomic

hybridization studies on six of the cases demonstrated a low overall frequency of genomic copy number changes, Together, these data indicate that mucinous cancers of the breast do not have the extensive genomic alterations that are typically found in more common variants of breast cancer. Thus, mucinous cancers most likely have less genetic instability than most other forms of breast cancer and the molecular pathogenesis of this form of breast cancer is likely to be substantially different than that of usual ductal breast cancer.

Lobular carcinoma: Although we have not studied lobular carcinoma cases, recent molecular pathways lobular as well as ductal carcinoma and their possible association will be briefly reviewed in the lecture.

LOH in the clonal evolution of flat colorectal neoplasms:

In contrast to invasive colorectal carcinomas that develop in typical exophytic adenoma-carcinoma sequences, some invasive cancers may evolve from flat mucosal dysplastic lesions. Despite their relatively small size, these flat colorectal lesions are often associated with high-grade dysplasia and may show an aggressive clinical course.

LOH patterns were detected in one of two forms: (i) homogeneous LOH throughout the microdissected foci, which indicated the early acquisition of LOH; and (ii) heterogeneous LOH, which were detected in a part of analyzed foci. The heterogenous LOH patterns observed in different portions of dysplasias-carcinoma in situ and invasive cancers in individual cases identified several different genetic patterns of tumour progression, either with linear or branching (divergent) trees. Homogeneous LOH was detected most frequently on 17p (68%) followed by 18p (53%), 18q (53%), 22q (34%) and 12q (27%). The average fractional allelic loss (FAL) for heterogeneous and homogeneous LOH was 0.57 and the average FAL for homogeneous LOH was 0.37 (significantly higher than the conventional type advanced colon cancer). Together, early flat-type colorectal tumors frequently shows the early occurrence of multiple LOH including 17p, 18p, 18q and 22q, which is coupled with additional LOH of other loci either simultaneously or in the early clonal progression phase. The extent and sequences of LOH may be the mechanisms responsible for the aggressive clinical behaviors of these tumors

Frequent hypermethylation of RASSF1A in early flat-type colorectal tumors:

We found that promoter hypermethylation of RASSF1A (the gene on 3p21.3) is a frequent event and it may start early in the background normal mucosa in this tumor type. An alternative cascade of abnormalities in RAS transduction pathways (low frequency of K-ras mutations) may be partly responsible for the flat morphology and aggressive nature of flat colorectal neoplasms.

Microsatellite instability (MSI) is uncommon in breast cancer:

In some tumors, defects in mismatch repair enzymes lead to errors in the replication of simple nucleotide repeat segments. This condition is commonly known as microsatellite instability (MSI) because of the frequent mutations of microsatellite sequences. Although the MSI phenotype is well recognized in some colon, gastric, pancreatic, and endometrial cancers, reports of MSI in breast cancer are inconsistent. We reported our experience with >10,000 amplifications of simple nucleotide repeats in noncoding genomic regions using DNA from 267 cases of breast cancer, including cases that represent all major histological types of breast cancer. We rarely (10 reactions) found unexpected bands in amplifications of tumor DNA that were not present in amplifications of normal DNA. We also evaluated the simple nucleotide repeats in the transforming growth factor type II receptor, insulin-like growth factor type II receptor, BAX, and E2F-4 genes, which are frequently mutated in tumors with microsatellite instability. No mutations of these genes were found in any of the 30 breast cancer cell lines and 61 primary breast cancer samples examined. These results indicate that mismatch repair errors characteristic of the MSI phenotype are uncommon in human breast cancer.

Birt-Hogg-Dubé gene mutations in human endometrial carcinomas and gastric carcinomas with microsatellite instability:

Target genes of MSI and their mutation frequency are quite different according to cancers of different organs and different pathological types. Here, we show an example of evolution of MSI target gene mutation in the course of tumor progression.

Birt-Hogg-Dubé (BHD) syndrome is a rare form of autosomal dominantly inherited genodermatosis characterized by benign hamartomatous skin lesions named fibrofolliculomas, and an increased risk for developing pulmonary cyst/pneumothorax and various forms of renal cell carcinoma. Many of the patients harbour

insertion/deletion mutations in the hypermutable poly(C)8 tract in exon 11 of the BHD gene. This mutational hot spot is also reported to be a target of mutation in microsatellite instability (MSI) sporadic colorectal cancer. Cases of sporadic endometrial carcinoma were screened for MSI status, and mutations of the poly(C)8 tract in exon 11 as well as other coding exons of the BHD gene. The poly(G)8 tract of the BAX gene, the poly(C)8 tract of MSH6 were also assessed. 28% showed MSI. Mutations in the poly(C)8 tract of BHD were detected in five of the 39 MSI cases (12.8%). BAX gene mutation was detected in ten of the 39 MSI cases (25.6%). Four tumours showed both BAX and BHD mutations, and a significant positive association was found between mutations of the two genes. When multiple foci were microdissected and individually screened for mutation, BHD mutations were shown to have been acquired during tumour progression, after mutation of the BAX gene, in three of five cases. Taken together, these findings show that the BHD gene is a target gene in MSI endometrial carcinoma. However, its mutational frequency is lower than that of BAX, and BHD mutation tends to occur during neoplastic progression after the acquisition of mutations in another MSI target gene, BAX.

In the gastric cancer, we showed that the BHD gene is a rare target in MSI-high gastric cancer (15.8%), and BHD mutation tends to occur downstream in the mutational events of other major MSI-high target genes including BAX and TGFbetaRII.

Clonality analysis in synchronous uterine and ovarian endometrioid carcinoma:
Synchronous development of carcinomas in the endometrium and ovaries is a fairly common phenomenon, but distinction of a single clonal tumor with metastasis from 2 independent primary tumors may present diagnostic problems. To determine clonality and the occurrence of progression, We microdissected multiple foci from 17 cases of synchronous endometrioid carcinomas and studied loss of heterozygosity (LOH), microsatellite instability (MSI), and PTEN mutations. Altogether, 14 synchronous tumors were genetically diagnosed as follows: single clonal tumor, characterized by concordant genetic alterations in both tumor sites, including identical LOH, identical PTEN mutations, and/or identical sporadic allelic instability patterns (4 cases); single clonal tumor with genetic progression, homogeneous LOH or identical PTEN mutations in both tumor sites and progressive LOH in ovarian metastatic foci (2 cases); and double (7 cases) or triple clonal tumors (1 case), determined by discordant PTEN mutations, heterogeneous LOH, and/or discordant MI patterns. Thus, 35% of synchronous tumors were monoclonal, 47% were polyclonal, and 18% were undetermined. The favorable

prognosis of synchronous endometrioid carcinomas may be due to the occurrence of PTEN mutations in both independent and metastatic tumors, the MI-positive independent primary tumors, and the low frequency of LOH.

Frequent genetic heterogeneity in the clonal evolution of gynecological carcinosarcoma, esophageal carcinosarcoma, spindle cell gallbladder carcinoma and its influence on phenotypic diversity:

Carcinosarcomas of the uterus, ovaries, and fallopian tubes are highly aggressive neoplasms with incompletely understood histogenesis. For this study, we microdissected a total of 172 carcinomatous or sarcomatous foci from 17 gynecological carcinosarcomas and analyzed allelic status on chromosomal arms 1p, 1q, 3p, 4q, 5q, 6q, 8p, 9p, 10q, 11p, 11q, 13q, 16q, 17p, 17q, 18q, and 22q. With the exception of a single case with microsatellite instability, we found shared allelic losses and retentions among multiple individually dissected foci of each case, strongly supportive of the concept of a monoclonal origin for these neoplasms. In eight of these cases, we also found heterogeneous patterns of allelic loss at limited numbers of chromosomal loci in either the carcinomatous or sarcomatous components of the neoplasms. These heterogeneous patterns of allelic losses were consistent with either genetic progression or genetic diversion occurring during the clonal evolution of these neoplasms. In two cases, we found the specific patterns of genetic progression to be consistent with sarcomatous components of the neoplasms arising from carcinomatous components. We conclude that most of the gynecological carcinosarcomas have a monoclonal origin, and that genetic progression and divergence parallel the development of divergent phenotypes in these tumors.

We found similar genetic patterns in esophageal carcinosarcoma and spindle cell carcinoma of the gall bladder. Our data supported the concept that esophageal carcinosarcoma is derived from a single clone originating from a squamous cell carcinoma with both progression and divergence within individual tumors. In four of six cases, the genetic changes indicated that an original clone of a pure squamous cell carcinoma apparently acquired carcinosarcomatous or sarcomatous phenotype by successive genetic changes. Two cases of gallbladder spindle cell carcinoma were found to be derived from a single clone originating from a adenocarcinoma by successive genetic changes.

Genetic evolution of alpha fetoprotein producing gastric cancer:

Alpha fetoprotein (AFP) producing gastric cancer is an unusual form of aggressive adenocarcinoma with a complex histological picture, including enteroblastic and hepatoid differentiation in addition to the ordinary adenocarcinoma component. We found that AFP-GC arises as an aggressive clone with extensive LOH including 17p, followed by 13q, 3p, 5q and 9p, 11q, 18q, 16q, and 8p. and high fractional allelic loss. Heterogeneous patterns of LOH indicated genetic progression and/or divergence in clonal evolution. Furthermore, in some cases 13q LOH was restricted to immunohistochemically AFP positive neoplastic foci. The presence of heterogeneous patterns of LOH suggested that the AFP producing carcinoma foci might evolve through genetic progression and/or genetic divergence. Silencing of the crucial gene on 13q may be involved in the acquisition of the AFP producing phenotype.

In the presentation, I mostly focused on our previous works, but I hope that the audience understand the importance of topographical microdissection and subsequent genetic analysis for various tumors. More new techniques should also incorporate the methodology.