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ORIGINAL ARTICLE

Profiling chromosomal-level variations in gastric malignancies

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Abstract

Aneuploidy arises from persistent chromosome segregation errors, or chromosomal instability. Although it has long been known as a hallmark of cancer cells, reduced cellular fitness upon induced ploidy alterations hinders the understanding of how aneuploidy relates to cancer development in the body. In this study, we used FISH analysis targeting centromeres to indicate ploidy changes, and quantitatively evaluated the ploidy statuses of gastric tumors derived from a total of 214 patients, ranging from early to advanced disease. We found that cancer cells reveal a marked elevation of aneuploid population, increasingly in cases diagnosed in advanced stages. The expansion of the aneuploid population is well associated with p53 deficiency, consistent with its essential role in genome maintenance. Comparisons among multiple locations within the tumor, or between the primary and metastatic tumors, indicated that cancer cells mostly retain their ploidy alterations throughout primary tumors, but metastatic tumors may consist of cells with either increased or decreased levels of aneuploidy. We also found that a notable proportion of polyploid cells are often already present in chronic gastritis epithelia. These observations underscore that chromosome-level variations are widespread in gastric cancers, shaping their genetic heterogeneity and malignant properties.

KEYWORDS
aneuploidy, cancer progression, DNA FISH, gastric cancer, heterogeneity

1 | INTRODUCTION

Chromosomal instability is a property in which cancer cells persistently fail to segregate chromosomes equally into daughter cells as they divide. This aberrant feature generates a spectrum of aneuploid cells that contributes to genetic intratumor heterogeneity and to cancer evolution through selecting a favorable karyotype for proliferation.[1–3]

Aneuploidy has been known as a hallmark of cancer cells since the late 19th century,[4,5] and several studies have attempted to elucidate

Abbreviations: AS, aneuploidy score; CIN, chromosomal instability; EBER-ISH, EBV-encoded small RNA-in situ hybridization; EBV, Epstein–Barr virus; FFPE, formalin-fixed paraffin-embedded; GCLS, gastric cancer with lymphoid stroma; HS, heterogeneity score; IHC, immunohistochemistry; ISH, in situ hybridization; MMR, mismatch repair; MSI, microsatellite instability; NGS, next-generation sequencing; RTK, receptor tyrosine kinase; SCNA, somatic copy-number alteration; TMA, tissue microarray.

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how aneuploidy contributes to tumorigenesis through the cultured cancer cell lines and mouse models.[6–8] However, experimental aneuploid models have not fully recapitulated the pathological conditions in human cancers.[9] The artificially induced aneuploid cells show a reduced proliferation rate in vitro, and when they were inoculated into mice, they showed decreased levels of tumor formation.[10–12] The loss of cellular fitness in aneuploid cells were thought to be attributable to proteotoxic stress arising from deregulated protein expression.[13–15] These seemingly contradictory observations between experimental models and clinical cancers have long been known as the "aneuploidy paradox."[16–18] Hence, a rigorous assessment of the genetic landscape at the chromosome level in clinical samples would offer a better understanding of the relevance of aneuploidy.

Next-generation sequencing-based approaches in patient-derived cell lines, organoids, and xenografts have attempted to elucidate the correlations between aneuploidy and pathological features in wide ranges of human cancers: human cancer consists of heterogeneous aneuploid cells, and specific ploidy patterns associate with cancer types.[3,19–22] However, to investigate how the ploidy alterations associate with cancer development and progression requires the interrogation of a wide range of patient-derived samples with thorough histological examinations.

Conventional NGS-based deep sequencing analyses provide focal genomic information, instead of larger changes encompassing chromosome arms or whole chromosomes. Various algorithms for NGS-based analyses allow for prediction of arm- or whole chromosome-level SCNAs,[23] and has indicated that most if not all cancers indeed reveal SCNAs as well as focal copy-number alterations.[24] However, tumors comprised of heterogeneous cell populations require single cell-based analysis, in which SCNAs are not readily analyzed by NGS-based approaches. Fluorescence in situ hybridization analysis allows the evaluation of genome structure, and has been used to detect oncogenic gene amplifications or fusions on a single cell basis.[25–27] The FISH analysis is also a versatile approach to infer ploidy alterations, by taking probes to centromeres. Its single cell-based analysis merits investigating the relevance of ploidy changes with respect to the histological landscape of cancer. In this work, we quantitatively evaluated the ploidy status of gastric cancer tissues, from "precursors" to advanced lesions, by using FISH targeting centromeres. We found that cancer cells are aneuploid, and higher level of aneuploidy associates with tumors of later-stage diagnosis and with poorer prognosis in advanced disease.

2 | MATERIALS AND METHODS

2.1 | Patients and data collection

Surgical specimens from patients who enrolled in the Cancer Institute Hospital of the Japanese Foundation for Cancer Research between 2008 and 2019 were retrospectively evaluated in this study. Medical records of the patients were reviewed to obtain the clinical datasets, including metastasis, recurrence, overall survival, and outcomes. Histological examinations of H&E stained specimens were carried out by certified pathologists and diagnosed based on the Japanese Classification of Gastric Carcinoma.[28] This study was approved by the institutional review boards of the Cancer Institute of the Japanese Foundation for Cancer Research (registry number: 2013–1128).

2.2 | Tissue microarrays

Representative lesions of respective surgical specimens were assembled on glass slides, and a series of TMAs were subjected to FISH, EBER-ISH, ISH, IHC, and standard H&E staining.

2.3 | Cell culture

Cell lines including RPE1, HeLa Kyoto (a gift from S. Narumiya, Kyoto University), and U251 were cultured in DMEM supplemented with 10% FBS. Cells were maintained at 37°C with 5% CO_2.

2.4 | DNA FISH

The FFPE tissues, including TMAs, were sectioned at thickness of 6 μm and placed on slides. These specimens were heated at 65°C for 2 h, deparaffinized in xylene for 10 min, and dehydrated with 100% ethanol. The specimens were treated for 30 min with pretreatment solution (K8005; Dako) at 95°C to improve the accessibility of DNA, and were proteolyzed in 50 mM Tris–HCl (pH 7.6) with protease K at 37°C for 25 min, which was deactivated with 50 mM $MgCl_2$ for 10 min at room temperature. The slides were then postfixed in 10% neutral buffered formalin for 2 min, and dehydrated in a graded ethanol series.

A mixture of FISH probes was applied onto the specimens, overlayed, and sealed with cover slips. The slides were denatured at 75°C for 5 min, and hybridized at 37°C for 48 h. After removing the cover slips, the slides were washed in 2× SSC (pH 7.0) with 0.3% NP-40 at 72°C for 2 min and were rinsed in 2× SSC. Slides were mounted with a medium containing DAPI. The DNA FISH probes for the centromeres of chromosome 6 and 16 were purchased from Vysis (CEP 6 [D6Z1] SpectrumOrange Probe and CEP 16 [D16Z3] SpectrumGreen Probe).

Cultured cells including RPE1, HeLa, and U251 cells were fixed with 3:1 methanol : acetic acid, dropped onto glass slides, and dried. The remaining steps of DNA FISH were followed by processes as described above.

2.5 | Slide imaging and analysis of FISH

The FISH images that were specified by their corresponding H&E-stained specimens were captured at 100× magnification objective in Z-stacks (six images at 1 μm intervals) using Keyence BZ-X700 (or 800) fluorescence microscopy. The Z-sectioned images were projected for visualizing the maximum intensities of FISH signals, and

the signals were counted for each cancer cell. One hundred cells of each case were examined. Scores for aneuploidy and heterogeneity were calculated as described previously.[29]

2.6 | Quantification of inter-distances of DNA FISH doublet signals

The FISH images were acquired at 100× magnification objective with an AxioImagerM1 microscope (Zeiss) equipped with a PRIME BSI sCMOS camera (Photometrics) in Z-stacks (six images at 1 μm intervals). Captured images were stacked with maximum intensity projection and interdistances between two adjacent signals were measured by Fiji software.

2.7 | Classifying molecular subtypes

Identification of EBV or MSI subtype of gastric cancer tissues was carried out as previously described.[26] Briefly, EBV subtypes were determined by EBER-ISH with BOND Ready-to-Use ISH EBER probe (PB0589; Leica), and ISH with the automated BOND system (BOND-MAX and BOND-III systems; Leica). For determination of MSI subtypes, IHC of MMR proteins including MLH1, MSH2, MSH6, and PMS2 was carried out, and tumors with the absence of at least one MMR protein were determined as MMR-deficient, MSI subtypes. Deficiency of p53 was determined by IHC, and classified into either proficient or deficient (accumulated or absent) depending on the level of expression of p53 protein. The expression levels of p53 in normal epithelial cells and lymphocytes were used as an internal control for the evaluation. The following Abs were used: MLH1 (ES05, NCL-L-MLH1; Leica), MSH2 (G219-1129, 556,349; BD Biosciences), MSH6 (EPR3945, GTX62383; GeneTex), PMS2 (A16-4, 556,415; BD Biosciences), and p53 (clone DO-7, M7001; Dako).

2.8 | Statistical analyses

Student's *t*-test was used to analyze the association between aneuploidy or heterogeneity scores and pathological findings. Regression analysis was used for the comparison of aneuploidy or heterogeneity scores and the period of recurrent metastasis. Overall survival was estimated with the Kaplan–Meier method, and Wilcoxon rank sum test was used to compare the groups. All statistical analyses were undertaken using JMP version 16 or GraphPad Prism 9.

3 | RESULTS

3.1 | Fluorescence in situ hybridization-based assessment of ploidy of human tumors

To assess the cellular ploidy status in human tissue, we analyzed patient-derived FFPE specimens by DNA FISH. Centromere-targeting probes were used as centromeres would in principle reflect ploidy status. We first specified a region of interest in H&E-stained sections that is rich in characteristics of cancer, such as high cell density and nuclear atypia. We then undertook side-by-side DNA FISH for the corresponding region, and examined cancer cells for DNA FISH signals (Figure 1A). For each specimen, DNA FISH results were plotted and summarized on a heatmap, in which the frequency distribution and thus degree of aneuploidy can be readily indicated (Figure 1B).

Cells that underwent DNA replication contain pairs of sister chromatids, which can be recognized as two signals adjacent to each other. When the interdistance of two adjacent signals is less than 0.4 μm, they often became inseparable; thus, we considered that these signals originated from one chromatid to minimize overestimating the chromosome number (Figure S1A). Conversely, in a sectioned tissue, the whole nucleus is not always preserved and some are severed, which could be a source of underestimation. To estimate the extent of signal counts that we may lose, we undertook DNA FISH analysis on cultured cells either fixed in solution or formalin-fixed and sectioned from a paraffin-embedded block, similar to sectioned tissue samples (Figure S1B,C). We tested for normal diploid RPE1 cells, near-triploid HeLa cells, and highly aneuploid U251 cells. When the numbers of DNA FISH signals in cells fixed in solution largely revealed their expected ploidy statuses, and when those in FFPE samples reduced, their chromosome number ranged by 14%–35%. These examinations indicated that our FISH-based ploidy assessment would provide an underestimated, but not an overestimated, measurement from the virtual ploidy status.

3.2 | Ploidy of cancer cells deteriorates in advanced disease

Despite the inherent technical limitations raised above, the advantage of FISH analysis is that it can be combined to geographic information of cells and allows to address how the ploidy alteration might contribute to invasion of cancers. We examined 190 gastric cancer patient-derived FFPE specimens, which included 63 cases of early cancer and 127 cases of advanced cancer, according to the Japanese classification of gastric carcinoma[26] (Figure S2A,B). The averaged karyotype distributions of all the cases on heatmaps indicated an apparent difference between early and advanced cancers: even with a wide ploidy distribution, there still is an enrichment of cells having two chromosomes in early cancers. By contrast, this enrichment was unclear and a higher extent of aneuploidization was evident in advanced cancers (Figure 2A). To quantitatively analyze these results, we applied two indices for cellular heterogeneity (HS) and aneuploidy (AS): HS reflects variability in chromosomal number states, and AS magnitude of chromosome number changes.[29] The differences in these parameters were largely discernible in karyotype distribution on heatmaps. In early cancers, the value of HS was closely proportional to that of AS. In advanced cancers, there were cases with higher AS, and the HS generally became less proportional to AS; the outliers indicate that aneuploid cells were propagated with increasing or decreasing heterogeneity (Figure 2B).

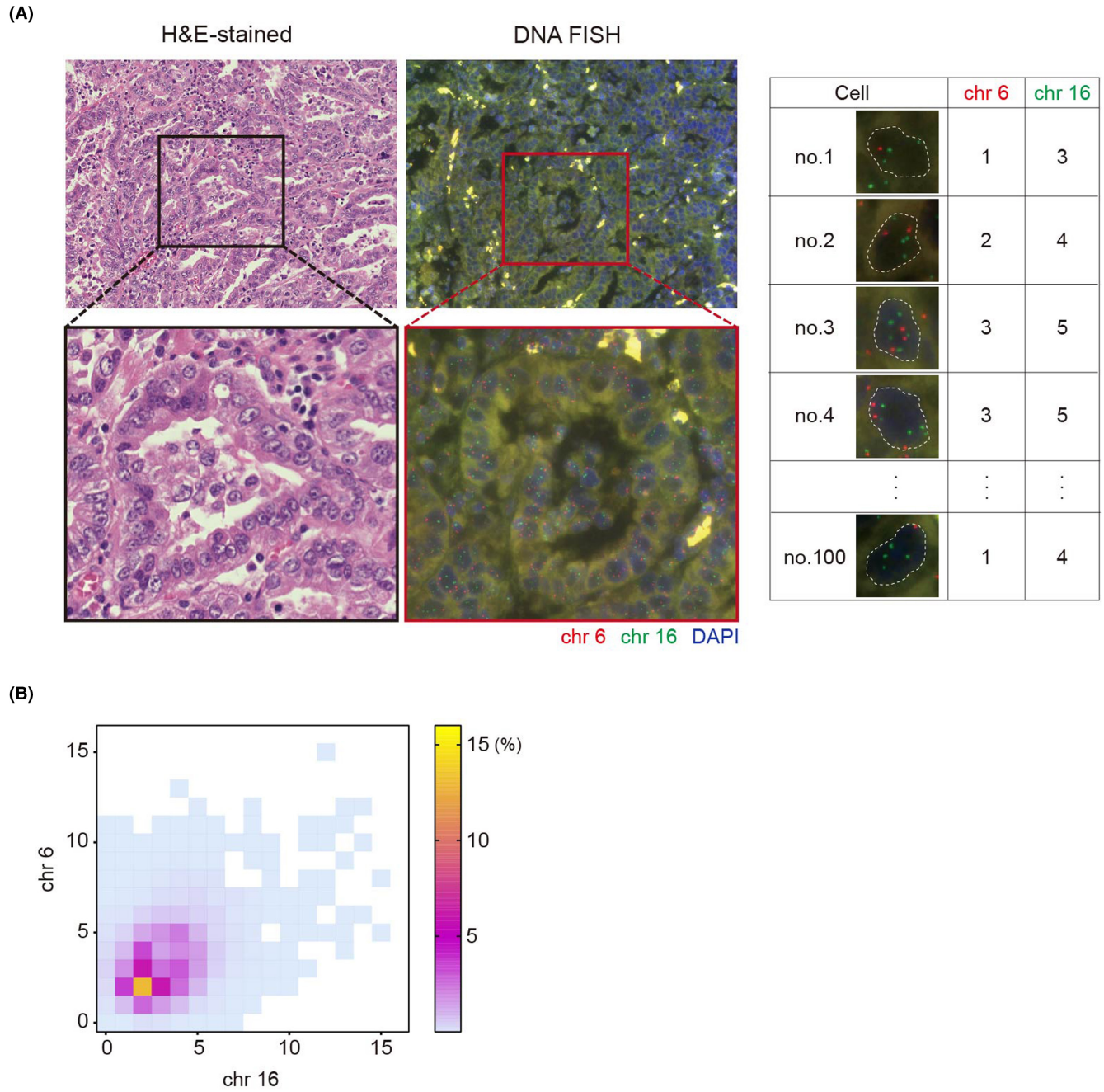
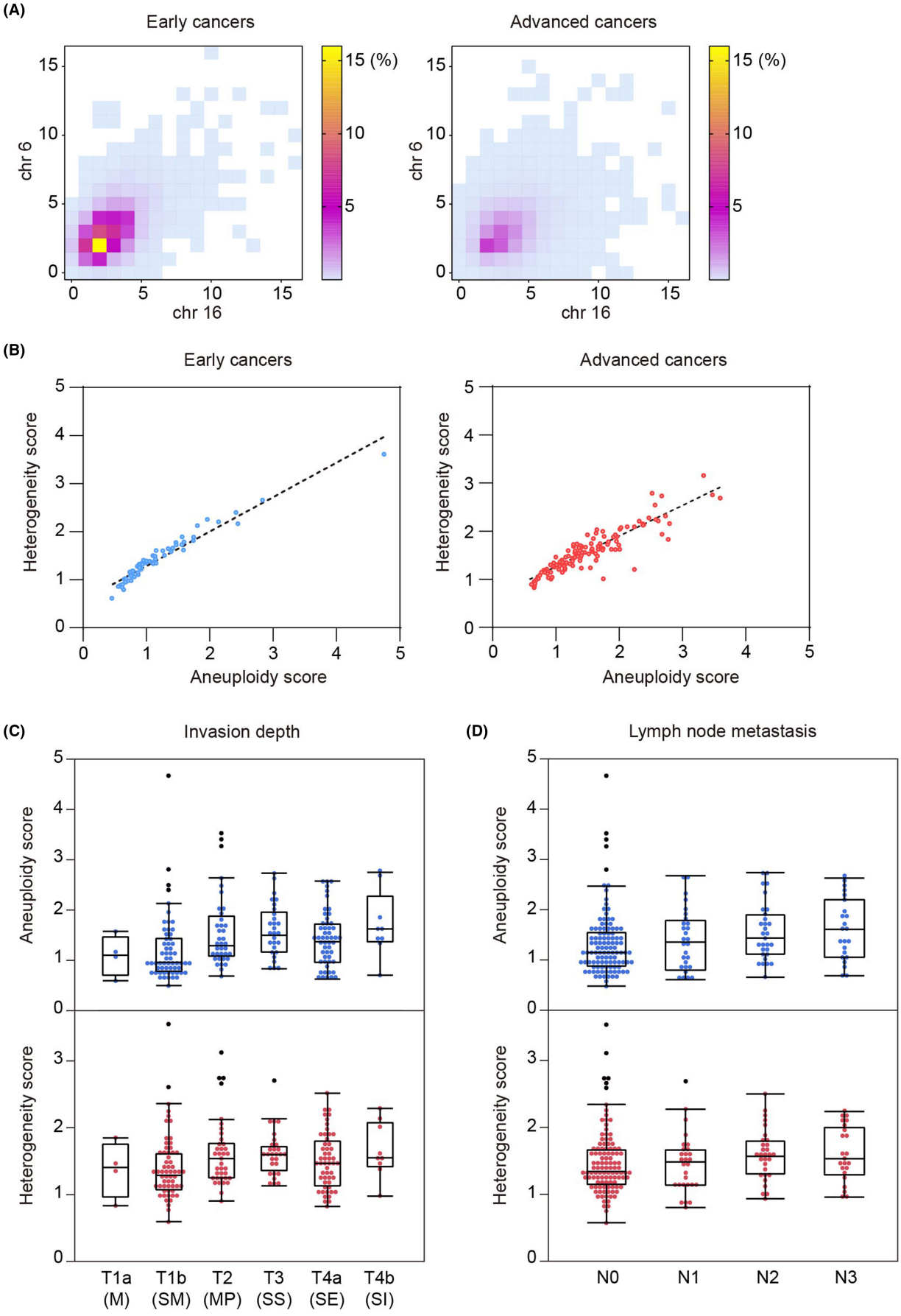


FIGURE 1 Fluorescence in situ hybridization-based assessment of ploidy of tumor cells. (A) A region of interest in DNA FISH specimen was specified on the basis of an H&E-stained specimen. One hundred cells in each case were examined and signals of centromeric probes, targeting chromosome (chr) 6 and 16, were counted. (B) Representative distribution of ploidy in a heatmap presentation. Proportion of cells with respective chromosome numbers is color coded

FIGURE 2 Ploidy alterations in early and advanced cancers. (A) Averaged cellular ploidy statuses of early cancers (63 cases) and advanced cancers (127 cases) were plotted onto heatmaps. The proportion of cells with respective chromosome numbers is color coded. (B) Scatterplots of heterogeneity and aneuploidy scores. Linear trend lines of early and advanced cancers are shown in both plots (early, $y = 0.716x + 0.5708$; advanced, $y = 0.6331x + 0.6357$). The coefficient of determination, r^2 values, of early and advanced cancers were 0.9427 and 0.8059, respectively. (C) Heterogeneity and aneuploidy scores relative to invasion depths of primary tumors, which were categorized according to the Japanese classification of gastric carcinoma: T1a(M), tumor confined to the mucosa; T1b(SM), tumor confined to the submucosa; T2(MP), tumor invading the muscularis propria; T3(SS), tumor invading the subserosa; T4a(SE), tumor invading or exposed beyond the serosa; T4b(SI), tumor invading adjacent structures. (D) Heterogeneity and aneuploidy scores relative to lymph node metastasis. N0, no lymph nodule metastasis; N1, metastasis in 1–2 regional lymph nodes; N2, metastasis in 3–6 regional lymph nodes; N3, metastasis in 7 or more regional lymph nodes. chr, chromosome



To relate ploidy alterations with histological characteristics of tumors, we compared the extent of aneuploidy between the pathological elements (Figure 2C,D and Figure S2C). We found an increase of both AS and HS indices accordingly to the depth of primary tumors. Notably, there was a marked aneuploidization when tumors extended further into the muscle wall, muscularis propria, from those remaining in submucosa (Figure 2C). These observations imply that ploidy alteration of cancer cells might relate to growth into the solid muscle layer of the gastric wall.

To determine whether ploidy alterations might associate with invasiveness of tumors, we evaluated the ploidy statuses between different levels of metastasis (Figure 2D). We found that the more lymph nodes were positive for metastasis, the higher levels of AS and HS were scored. Similarly, higher aneuploid levels were attained in the presence of vascular or lymphatic invasion than its absence. Moreover, tumors in an advanced stage with a distant metastasis typically revealed high levels of aneuploidy (Figure S2C). These results suggest that elevated levels of aneuploidization coincide with acquirement of a spectrum of invasiveness.

3.3 | Correlation between ploidy alterations and clinicopathologic characterizations

Although advanced cancers on average revealed higher levels of aneuploidy than early cancers, they both consisted of cases with various degrees of aneuploidy. To address the prospective pathological significance of aneuploidization, we grouped advanced cases into high or low aneuploidy and compared the overall prognosis. The Kaplan–Meier curve indicated that the higher aneuploid group tended to associate with a poorer outcome (Figure 3A).

Histologically, poorly differentiated or moderately differentiated tubular adenocarcinoma dominated in advanced cases, irrespective of the aneuploid levels (Figure 3B). However, the high aneuploid group seemed more prone to invasion, suggested by the higher incidence of lymph node metastasis, venous invasion and invasion depth (Figure S3). These characteristics must have had negative effects on the clinical outcomes in the advanced cases. In early cancers, a significant number of cases were diagnosed as well-differentiated tubular adenocarcinoma. Remarkably, signet-ring cell carcinoma was found to be enriched in cases with low aneuploid, and "carcinoma with lymphoid stroma" in high aneuploid populations (Figure 3C). These histological subtypes were barely found as advanced disease, which might imply that they are less aggressive.

3.4 | Ploidy alterations in primary and metastatic tumors

As we observed the increased aneuploid cell populations in cases with advanced stages (Figure 2), we next determined the level of aneuploidy changes when cells infiltrate into the gastric wall. To address this, we examined the ploidy status of cancer cells in tumors with contiguous invasion to serosa, on specimens encompassing the whole layer of the gastric wall (Figure 4). In four cases examined, we found highly aneuploid cells throughout the tumor, and their distribution profile appeared largely consistent from the mucosal to serosal foci, for each case (Figure 4A). Interestingly, we noticed that the levels of heterogeneity were largely maintained yet slightly fluctuated with depth: case 1 showed a decreased HS with depth, and case 3 showed the opposite (Figure 4B). These observations suggest that the whole primary tumor in advanced stage in principle consists of akin aneuploid cells whose extent of aneuploidy could alter in various ways during proliferation.

Next, we sought to investigate how ploidy alterations might relate to cancer cells in relapsed tumors. To address this, we examined metastatic foci in distant organs, such as liver, lung, or ovary, and their associating primary tumors (Figure 5A). The karyotypic distribution of metastatic foci appeared to largely carry over from those of primary lesions, consistent with the possibility that metastatic tumors stem from the cells that inherited proper ploidy alteration of cells in primary tumors. With closer inspection and scoring the aneuploid population, we could identify discernible ploidy alterations between the primary and metastatic tumors. Remarkably, metastatic tumors to liver and lung contained cases with elevated levels of aneuploidy, and, conversely, ovary tumors with decreased levels (Figure 5B). Interestingly, we also found that cases with highly aneuploid cells in the primary lesions tend to relapse after shorter periods of time (Figure 5C). These observations allow us to reason that tumors with genetically heterogeneous populations have the advantage to yield cells that can better adapt and grow in a different microenvironment.

3.5 | Conditions that allow propagation of aneuploid cells

Based on The Cancer Genome Atlas project, gastric cancers have been categorized into four molecular subtypes,[30] thus we determined whether any of the categories are associated with aneuploidization. Among all 190 cases, 16 cases were found to be EBV-positive tumors, and 17 cases with deficient MMR indicative of MSI. The other two entities, genomically stable and CIN, are stratified by the degree of copy number variation of focal genomic reads. As these two groups are known to largely correlate with the statuses of p53 tumor suppressor, we grouped the rest by IHC staining for p53 instead: 85 cases were estimated to be p53 deficient (either accumulated or absent) and 72 cases p53 proficient (Figure 6A,B). We found that the p53 deficient cases revealed a marked increase of aneuploid populations compared to the other subgroups (Figure 6A). Given the pivotal role of p53 in genome maintenance, it is reasonable to find highly aneuploid cases enriched in this group. Importantly, these findings indicate that CIN literally categorizes cases with ploidy-level alterations, in addition to focal copy number changes detected in sequencing-based analyses.

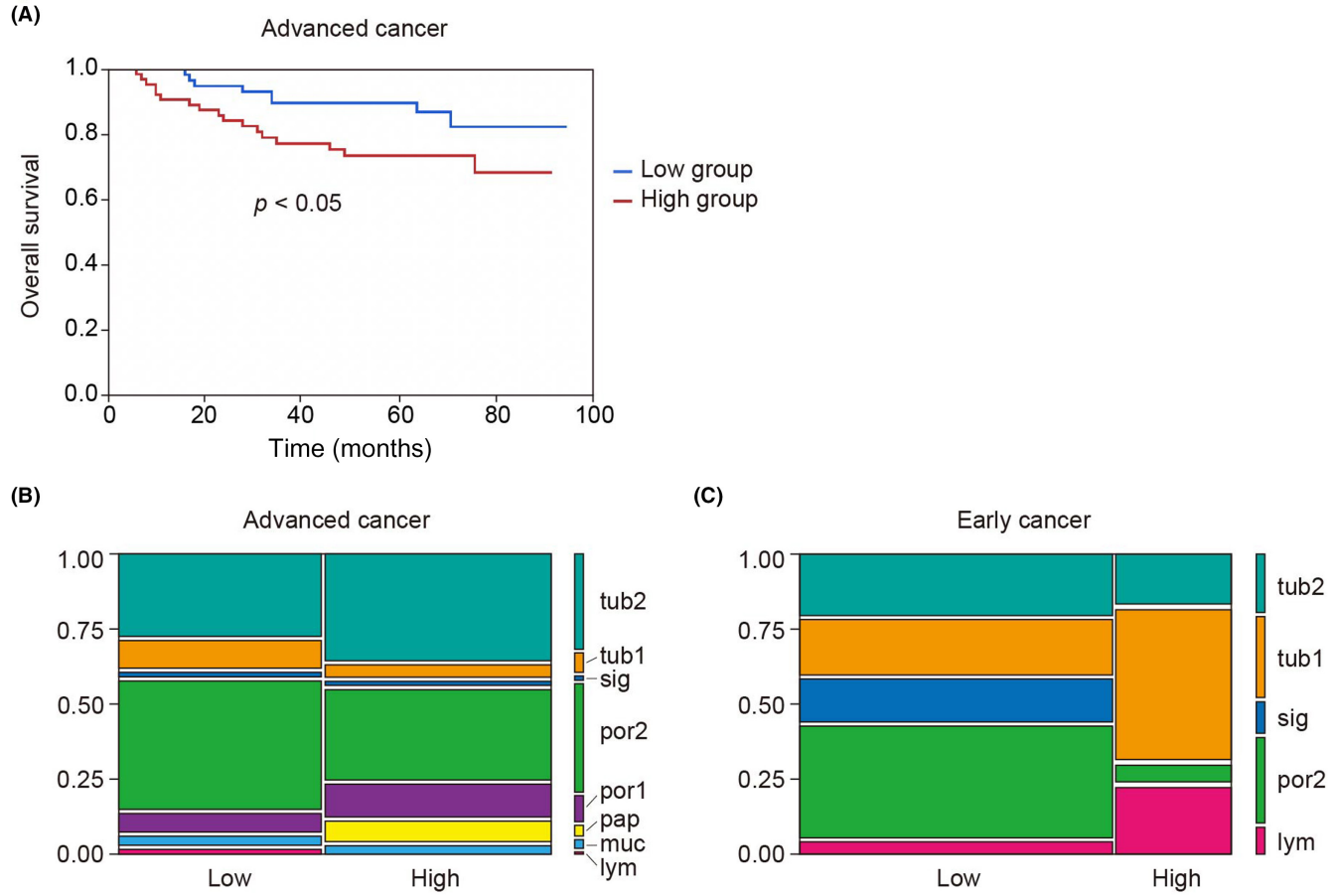


FIGURE 3 Correlation between ploidy alterations and clinicopathologic characterizations. (A) Kaplan–Meier curves of high and low aneuploidy groups in advanced cancers. The high aneuploidy group (60 cases) included cases with an aneuploidy score higher than 1.365, providing maximun difference in overall survival rates, and the low aneuploidy group included the remaining cases (67 cases). *p* values were obtained using Wilcoxon tests. (B,C) Mosaic plot showing the proportion of histological subtypes in high and low aneuploidy groups of (B) advanced cancer and (C) early cancer. High (17 cases) and low (46 cases) aneuploidy groups in early cancers were divided at AS 1.365 as in advanced cancers. lym, gastric cancer with lymphoid stroma; muc, mucinous adenocarcinoma; pap, papillary adenocarcinoma; por1/2, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma, solid/nonsolid type; tub1/2, well/moderately differentiated tubular adenocarcinoma

Deficiency of p53 is attributable to a mutation or silencing of the *TP53* gene, which is indicated by accumulated or absent expression of p53. To address which p53 status better accepts CIN in advanced disease, we compared the extent of aneuploidization between tumors with different levels of p53 expression (Figure 6B,C). Interestingly, tumors with absent p53 showed wider distribution of aneuploidy than those with accumulated p53, and the overall prognosis appeared to be poorer in the former cases than in the latter (Figure 6D,E). These observations in clinical samples are consistent with the widely accepted view that the levels of p53 expression might be correlated to the extent of tolerance to aneuploidy in cancer cells.[31–33]

3.6 | Emergence of polyploid cells in nonmalignant lesions

Another condition that is known to render cancers susceptible to propagating aneuploid cells is doubling the whole genome through tetraploidization.[34] Therefore, to address whether this ploidy change might contribute to generating highly aneuploid populations in gastric cancers, we studied a spectrum of gastric lesions, including chronic gastritis associated with intestinal metaplasia and adenoma (Figures 7 and S4). By contrast to epithelial cells in the foveolar epithelium of the normal mucosa, in which the vast majority were diploid, there was a spread of the aneuploid population including polyploidy in cells comprising adenoma or metaplasia (Figure 7A). An estimate of cell proportions also indicated that these lesions have decreased diploid, but increased polyploid cell populations (Figure 7B). These observations raise an interesting hypothesis that, with respect to ploidy alteration, there are proportions of epithelial cells in nonmalignant lesions that seem to be already on the pathological path toward carcinomas.

4 | DISCUSSION

We examined ploidy alterations in gastric cancer, which provided insights into the pathological relevance of aneuploidization

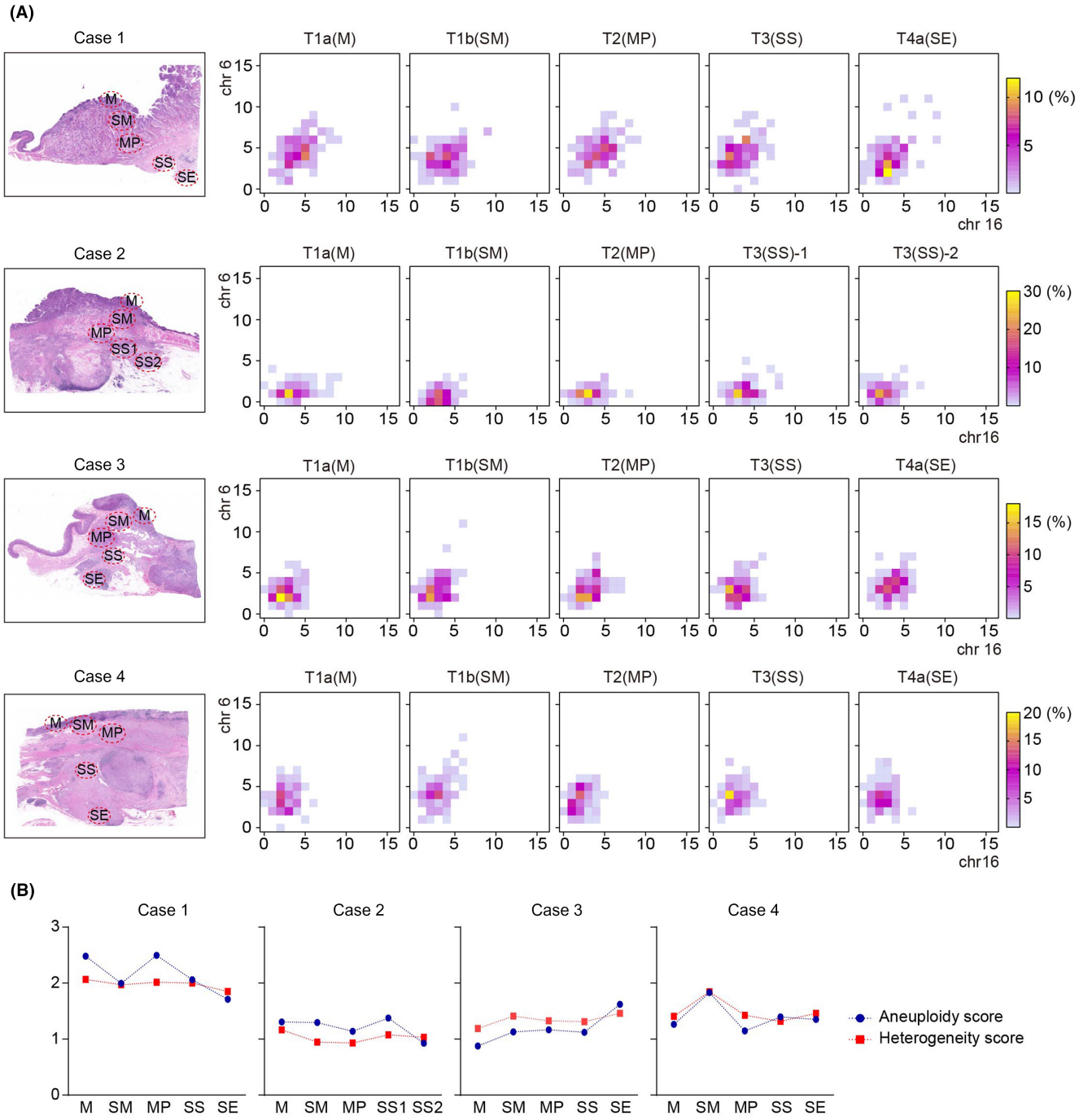


FIGURE 4 Ploidy alteration of cancer cells in massive tumors with contiguous invasion to serosa. (A) H&E staining of four cases and their corresponding heatmaps of cancer cells at different depths. (B) Aneuploidy and heterogeneity scores of tumor cases in (A). chr, chromosome; T1a(M), mucosa; T1b(SM), submucosa; T2(MP), muscularis propria; T3(SS), subserosa; T4a(SE), serosa exposed

in multiple contexts: tumor invasiveness, metastases, and lymphoid stroma reaction. In our DNA FISH analysis, we used two centromere-targeting chromosome probes, which allowed for a scoring of aneuploidy and heterogeneity in cell populations. We excluded chromosomes harboring amplification or deletion of genes that are known to be associated with gastric cancers, so that we could evaluate aneuploid distributions, irrespective of selection bias as much as possible.[30]

The level of aneuploidy and its heterogeneity were largely elevated in tumors in advanced stages (Figure 2A,B). Histologically, the difference between SM and MP distributions indicates that cells tend to acquire higher levels of aneuploidy when they infiltrate into the muscle layer (Figure 2C). These observations led us to speculate that aneuploidization might be causally related to an acquisition of further malignant phenotypes, including invasiveness.

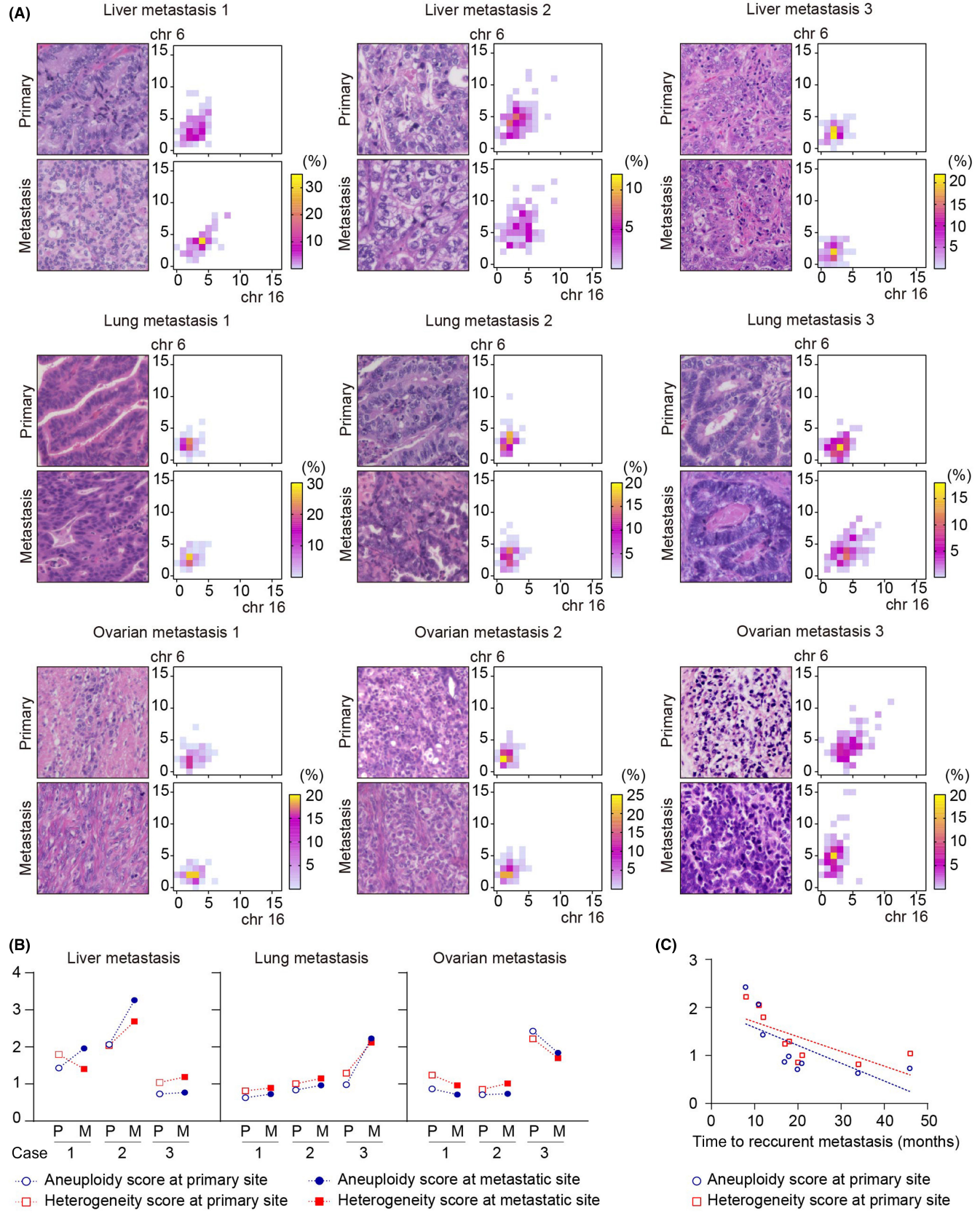
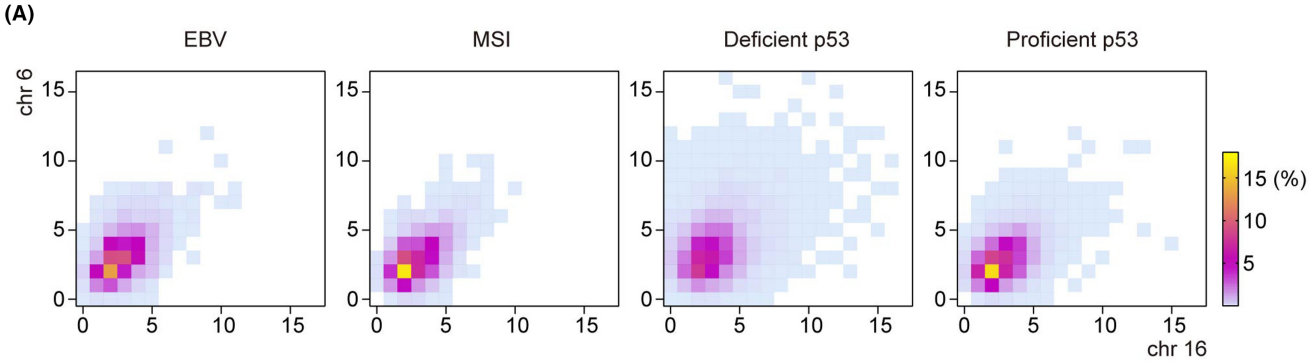
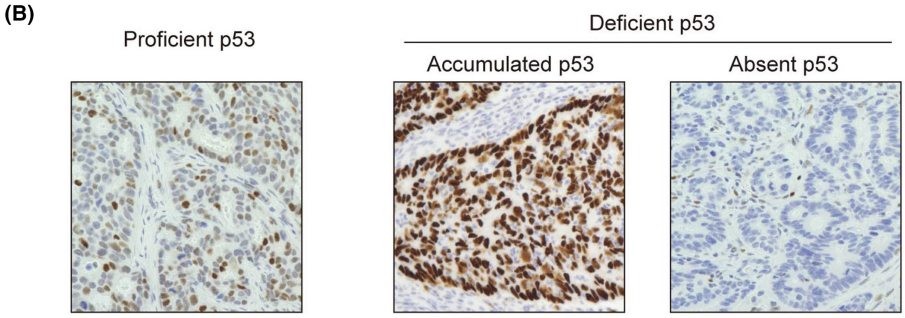
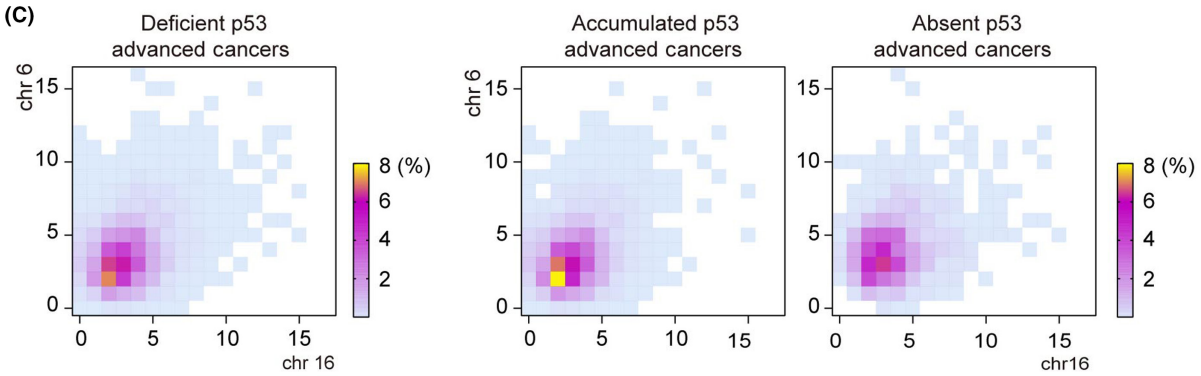
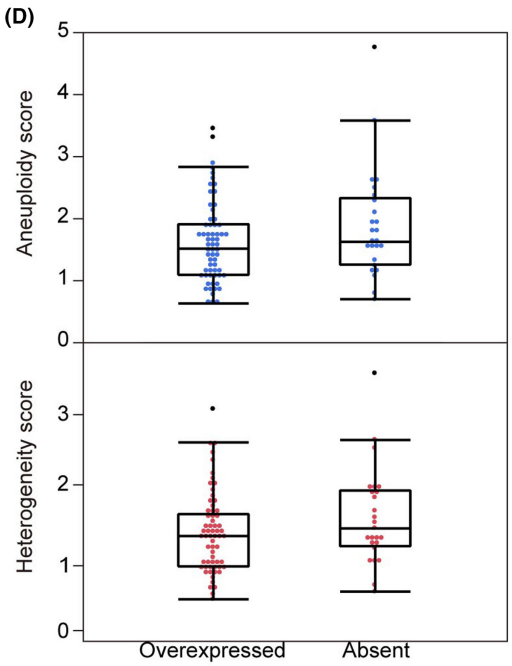


FIGURE 5 Ploidy alteration of cancer cells in relapsed tumors. (A) H&E staining of primary and metastatic tumors and their corresponding heatmaps. Three cases each of liver, lung, and ovarian metastatic tumors were analyzed with their primary tumors. chr, chromosome. (B) Aneuploidy and heterogeneity scores of primary (P) and metastatic (M) tumor pairs. (C) Correlation between aneuploidy or heterogeneity scores of primary lesions and the time to recurrent metastasis ($p < 0.05$)









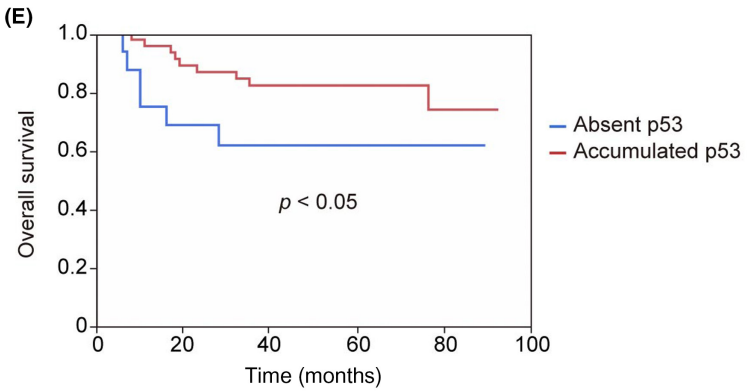


FIGURE 6 Propagation of aneuploid cells and related molecular backgrounds. (A) Heatmaps showing averaged cellular ploidy statuses of tumors with Epstein–Barr virus (EBV)-positive (16 cases), microsatellite instability (MSI) (17 cases), deficient p53 (85 cases), and proficient p53 (72 cases). (B) Immunohistochemistry for p53. Representative images of p53 proficient and deficient (accumulated or absent) tumors. (C) Averaged cellular ploidy statuses of advanced cancers with deficient p53 were plotted onto a heatmap (left). These advanced cancers with deficient p53 were further divided into accumulated p53 (63 cases) and absent p53 (25 cases) (middle and right panels), and the heatmap presentations are shown. chr, chromosome. (D) Aneuploidy and heterogeneity scores of tumors with accumulated and absent p53. (E) Kaplan–Meier curves of cases with accumulated or absent p53. *p* values were obtained using Wilcoxon tests

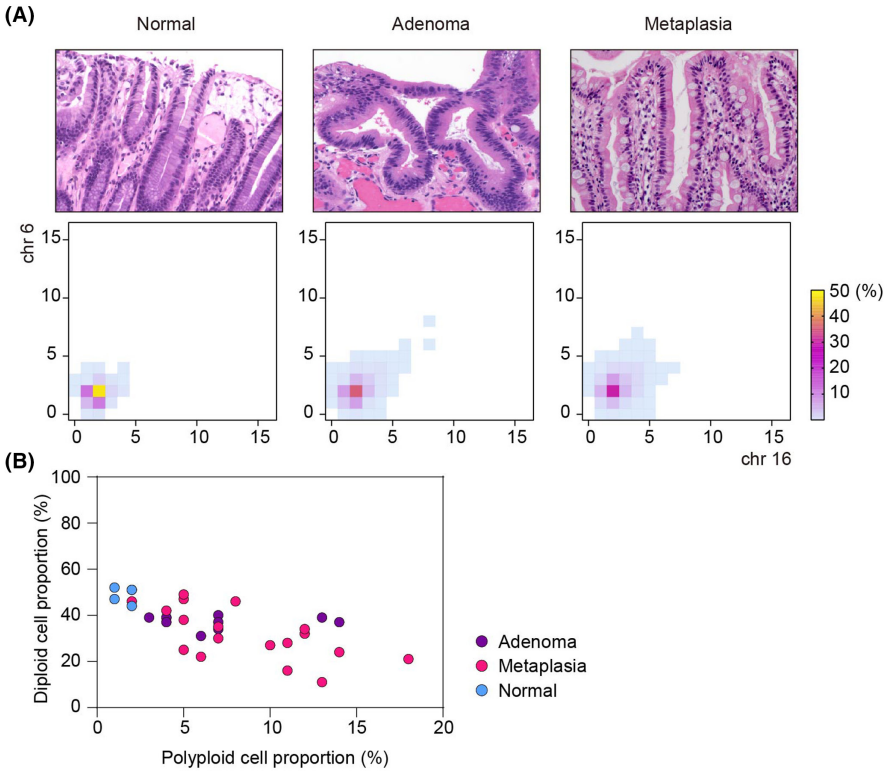


FIGURE 7 Emergence of polyploid cells in nonmalignant lesions. (A) Heatmaps showing averaged cellular ploidy statuses of foveolar epithelium of normal gastric mucosa (5 cases), intestinal-type tubular adenoma (10 cases), and intestinal metaplasia (18 cases). (B) Scatter plot showing the percentage of cells that are diploid and polyploid in cases analyzed in (A). chr, chromosome

Tumors spanning the whole layer of the gastric wall were composed of cells with largely similar levels of aneuploidy irrespective of depth. It is likely that propagation of highly aneuploid cells gave rise to the macroscopic tumor mass, even if there had been a stepwise increase of aneuploidy as cells infiltrated into the muscle layer in earlier stages. However, in some cases, the levels of aneuploidy were found to slightly fluctuate, either in an increasing or a decreasing manner (Figure 4B). These observations allow us to speculate that some tumors are composed of cells with increasing levels of aneuploidy as they grow, whereas some tumors of cells with selected ploidy patterns.

Of note, such fluctuations in ploidy status were also observed in distal metastatic recurrent foci (Figure 5A,B). In cases we studied, the levels of aneuploidy of metastatic tumors in liver or lung were rather increased, whereas metastatic tumors in ovaries showed a decreased extent of aneuploidy from their primary lesions. These differences in ploidy alterations could reflect the multifaceted nature of the invasion–metastasis cascade, and we can speculate two underlying possibilities. First, the extent of aneuploidization might be associated with preferable aneuploid levels for propagation of cancer cells at the microenvironment of a foreign, potentially inhospitable tissue: an increased level of aneuploidy is advantageous for cancer cells to proliferate in liver or lung, but not in ovary. Second, a more challenging possibility, is that the difference might originate from the process of invasion–metastasis: ovarian metastasis of gastric cancer arises through a direct, peritoneal seeding. Traveling along this route might require higher cellular fitness that can proliferate at the metastatic site, accounting for the decreases in aneuploidy levels. In contrast, metastasis to liver or lung is achieved by hematogenous or lymphatic spread. These paths might allow colonization of cells maintaining their original ploidy status at distant sites, and re-elevating the aneuploid level as they grow into a macroscopic tumor mass.

In addition to the possible association of the metastatic route with aneuploidization, chemotherapy, which is often combined with surgery, is reported to be associated with aneuploidization.[35] The early and advanced cancer cases we analyzed did not undergo chemotherapy before gastrectomy, that is, neoadjuvant chemotherapy. Among the metastatic tumors, however, three patients with ovarian tumors were treated either with XELOX (capecitabine and oxaliplatin), SOX (silicate-1 and oxaliplatin) or TS-1 (titanium silicate-1), and one patient who relapsed in liver and one patient who relapsed

in lung used TS-1 after surgery. Thus, metastatic tumors that underwent platinum-based chemotherapy seemed to show reduced extents of aneuploidy and heterogeneity, while DNA synthesis inhibitors did not. A possible implication from this limited number of cases is that subpopulations had been selected by chemotherapy, and such an effect seems to be dependent on the types of chemotherapy provided.

Stratification of tumors according to the difference in aneuploidy levels allowed us to determine that a histological subtype called GCLS was enriched in the high aneuploid groups of early cancers, and all of the GCLS subtypes were associated with EBV infection[36] (Figure 3C). Gastric cancer with lymphoid stroma histology is known to be rarely found in advanced stage (Figure 3B). The reactive lymphoid stroma is thought to prevent tumor spread and thus contributes to a favorable prognosis.[37,38] Analogous to MSI-high cancers, our observation raises an interesting possibility that highly aneuploid tumors, as found in the subset of EBV-associated gastric cancer, might trigger lymphocyte infiltration to compete against tumor growth (Figure 3B). If so, intervention using tumor immunity could be an indication for advanced tumors of not only the MSI subtype, but also the subset of CIN gastric cancers.

Extensive levels of aneuploidy were enriched in the p53-deficient cases, which underlines the significant role of p53 in chromosomal stability (Figure 6C). Intriguingly, advanced cases with absent p53 showed poorer prognosis on average than those with accumulated p53, which is indicative of p53 missense mutation (Figure 6E). Given that p53 mutation often acquires tumorigenic gain-of-function driver activities in a wide spectrum of cancers,[33] one scenario is that the tumor suppressive function of p53 might have a more prevalent role in other tumors of different tissue origins, including stomach. Alternatively, a more interesting implication from our observations is that the tumor suppressive function of p53 is primarily important to prevent propagation of aneuploid cells and leads to a more aggressive disease in its absence.

Is it solely p53 deficiency that causes a remarkable degree of aneuploidization? Tumors in the CIN subgroup, which is largely deficient of p53, are known to often associate with amplification of RTKs and can be sensitive to inhibition of the corresponding RTK signaling pathways. Therefore, we consider it plausible that activated RTKs can affect mitotic chromosome segregation machineries, and lead to an increase in aneuploid cell populations. These activated RTK-driven cells might vigorously proliferate in the absence of proficient p53.

Finally, the presence of increased polyploid cell populations in prospective precursor lesions, such as intestinal-type tubular adenoma and intestinal metaplasia, is worth noting, which prompted us to speculate that the ploidy alterations might precede tumor initiation (Figure 7). In classical pathological examinations, nuclear atypia, including enlargement of nucleus, is indicative of cells having malignant potential. If nuclear atypia reflects increase in ploidy, how polyploid cells might contribute to drive malignant transformation is an obvious question awaiting future investigations.

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DISCLOSURE

KT serves as a consultant to Nichirei Bioscience, holds a patent on Sysmex, receives lecture fees from Kirin Kyowa, and receives research funds from Fujireibio, Daichi Sankyo, and Sony. The other authors have no conflict of interest. KT and TH are Editorial Board Members of *Cancer Science*.

ETHICS STATEMENT

This study was approved by the institutional review boards of the Cancer Institute of Japanese Foundation for Cancer Research (Registry number: 2013–1128).

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SUPPORTING INFORMATION

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