Multiscale heterogeneity in gastric adenocarcinoma evolution is an obstacle to precision medicine

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Research Article

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Abstract

**Background:** Cancer is a somatic evolutionary disease, which adapts to environmental cues based on genetic constraints. Using multiregional exome sequencing, we tested the effect of somatic evolution on intratumoral heterogeneity and its putative clinical implications in adenocarcinomas of the stomach and gastroesophageal junction (GC).

**Methods:** The study comprised a prospective discovery cohort of 9 and a validation cohort of 487 GCs. Multiregional whole-exome sequencing was done using 48 tumor samples (range: 3-10 tumor samples/patient) of the discovery cohort.

**Results:** In total, the discovery cohort harbored 16,537 non-synonymous mutations (mutations/sample: median n=159; mutations/patient: median n=369). Intratumoral heterogeneity of somatic mutations and copy number variants were present in all tumors of the discovery cohort. 53-91% of the non-synonymous mutations were not present in each patient's sample; 399 genes harbored 2-4 different non-synonymous mutations in the same patient; 175 genes showed copy number variations, the majority being heterogeneous, including CD274 (PD-L1). Phylogenetic analyses provided evidence for branched evolution being the most complex in a microsatellite instable GC. The analysis of the mode of evolution showed a high degree of heterogeneity in deviation from neutrality within each tumor. Studies on the validation cohort showed that the subclonal loss of SMAD4 is an independent predictor for poor patient outcome.

**Conclusions:** Neutral and non-neutral somatic evolution shape the mutational landscape in GC. It leads to complex spatial intratumoral heterogeneity and may have profound effects on patient management. It provides crucial information for an individualized understanding of clinical prognosis and therapeutic options in GC patients.

**Highlights**

- We provide evidence of a substantial genetic and spatial intraprimary heterogeneity of adenocarcinomas of the stomach and gastroesophageal junction.
- The assessment of clonality depended on the number of samples studied and the extent of intratumoral heterogeneity.
- We found evidence of both a neutral and a non-neutral cancer expansion model, depending on the patient and on the sample.
- Pathway analyses showed marked intratumoral heterogeneity. Different pathways are effective during cancer development and progression, pointing towards interdependency and somatic evolutionary trajectories: cancer progression in TP53 mutant GCs is linked to subsequent (putative subclonal) alterations in the TGFβ- and SWI/SNF pathway.
- Our data strongly argue against the application of companion diagnostics to a single tissue specimen or specimens obtained only from the primary tumor.

**Introduction**
Gastric cancer (GC) is the fifth most common cancer in the world.\textsuperscript{1} In Western countries, the prognosis is dismal due to diagnoses in advanced disease stages often limiting therapeutic options. Compared with non-small cell lung cancer, targeted palliative therapeutic options are still limited in GC,\textsuperscript{2} though they demonstrated significant efficacy in a more recent umbrella trial.\textsuperscript{3}

An integrative genomic analysis carried out by Cancer Genome Atlas Research Network aimed to provide a roadmap for patient stratification and trials of targeted therapies, and lead to a proposal of four molecular subtypes, which were: Epstein-Barr virus-associated (EBV), microsatellite unstable (MSI), chromosomal unstable (CIN) and genomically stable (GS) GC.\textsuperscript{4} However, various validation studies conducted by ourselves and other research groups lead to the identification of a marked intratumoral heterogeneity, which stands to compromise the development and usage of targeted therapies in GC.\textsuperscript{5, 6, 7} We found evidence of intratumoral heterogeneity for e.g. HER2 status,\textsuperscript{8} MSI status\textsuperscript{9} and \textit{PIK3CA} genotype.\textsuperscript{10}

These observations suggest that processes of somatic evolution, i.e. mutation and selection of a tumor cell population on a time scale of months and years, are an important part of the biology of GC. Different tumor subclones coexist and substantially contribute to genetic and phenotypic diversity. The mechanism, by which patterns of intratumoral heterogeneity are generated, is largely unknown. As a null hypothesis for heterogeneity, a neutral model where mutations have no fitness effects was developed by Williams et al.\textsuperscript{11} In the neutral model, cancers acquire all tumor-driving alterations responsible for cancer expansion in the first malignant cell.\textsuperscript{11} Thereafter the cancer expands and neutral variation is generated, reflected by a large number of (probably non-functional) passenger mutations that are responsible for the extensive and common intratumoral heterogeneity. In the non-neutral cancer model, ongoing clonal selection and adaption to microenvironmental niches “shapes” the intratumoral genetic heterogeneity having a strong role during cancer growth. Williams et al.\textsuperscript{11} analyzed 14 tested types of cancers and found that GC showed most agreement to the neutral cancer model, with more than 40% of the tumors supporting neutral evolution. However, these findings were based on single sample analyses and merit validation using different samples from the same primary tumor by multiregional sequencing.

We hypothesize that the individual trajectory of somatic evolution, i.e. temporal/phylogenetic order of mutations, is an important determinant of the individual clinical behavior of GCs, which needs to be integrated into future clinical decision making. To test this hypothesis, we carried out multiregional whole exome sequencing analyses of primary GCs providing evidence for substantial intratumoral and intermetastatic heterogeneity, which applies to variant allele frequencies, the type of single nucleotide variation, copy number variation, and we also provide evidence of a neutral and non-neutral cancer expansion model in GC.

**Results**

\textit{Patient cohort for whole exome sequencing (discovery cohort)}

The clinicopathological characteristics of the discovery cohort are summarized in Table 1. A total of 45 samples were obtained from the primary tumors (3 to 7 samples per case; Table 1; Figure 1). In a single case, three samples were collected from three separate lymph node metastases. Including the non-neoplastic stomach mucosa, we finally obtained whole exome sequences from 57 tissue samples (4 to 11 samples per case). The
median sequencing coverage was 163x for tumor samples and 170x for non-neoplastic mucosa (Suppl. Table 3). Median tumor purity (computationally assessed by Sequenza) was 33% (12%-98%) (Suppl. Table 7). Raw sequencing data were deposited at the European Genome Archive (EGAS00001004525).

**Somatic signatures**

We analyzed the observed somatic signatures in their 5’ and 3’ base context thus resulting in 96 possible mutation types. Based on the exome data, we retrieved the individual mutational signature from each tumor sample in relation to the non-tumorous tissue. We found that the somatic signatures of GCs were consistent with previous reports: signature 1 was found in 48 tumor samples (100%), signature 15 in 48 (100%), signature 18 in 29 (60.4%), signature 10 in 3 (6.3%) and signature 29 in 2 samples (4.2%).

**Non-synonymous somatic mutations**

The results of the whole exome sequencing of the discovery cohort are summarized in Suppl. Table 4-10. Results from the independent validations of SNVs using Sanger sequencing, pyrosequencing or ddPCR™ demonstrating concordance are presented in Suppl. Table 2.

The discovery cohort harbored 16,537 non-synonymous mutations (i.e. missense, nonsense and frameshift; Suppl. Table 4), which were unevenly distributed among patients and patient samples (Figure 2). In the individual patient, the number of non-synonymous mutations ranged from 181 to 3111 (median: 369), and in the individual tumor sample, it ranged from 49 to 2348 (median: 159). The highest mutational burden was found in the MSI GC, with regard to patient (n=3111) and sample (n=2348).

The non-synonymous mutations were not evenly distributed among the patient samples. Between 8.7% and 46.8% of the non-synonymous mutations were found in all samples of the same patient (= “homogenous” distribution; Table 1). The vast majority of non-synonymous mutations (53.2-91.3%) was not present in each sample of the patient (= “heterogeneous” distribution). With regard to all non-synonymous mutations (n=4413 genes), mutations in 1642 (37.2%) genes were homogenously distributed and for 3272 (74.1%) genes they were heterogeneously distributed (Suppl. Table 8). Interestingly, mutations in 501 (11.3%) genes were both, homo- or heterogeneously distributed, albeit in separate cases (Suppl. Table 8).

The “spatial” heterogeneity of the mutational landscape was further diversified by genetic heterogeneity: 398 genes harbored two to five different non-synonymous mutations in the same patient (Suppl. Table 9). Again, the highest number of “multiple” non-synonymous mutations/genes was found in the MSI GC (320 genes with ≥2 mutations). The prevalence of genes with ≥2 non-synonymous mutations varied from 3 to 320 per patient and was also not evenly distributed among the different samples (Suppl. Table 9). The different mutations were present in the same as well as in different patient samples.

In a single case sequence data were obtained from three separate lymph node metastases. Similarly, the number and type of non-synonymous mutations varied between each lymph node metastasis and between the primary tumor and the three lymph node metastases. Interestingly, 172 mutations present in the primary were not detected in the lymph node metastases, while 58 mutations found in the lymph node metastases were absent in the samples obtained from the primary tumor (Suppl. Table 10).
Collectively, these data provide evidence that adenocarcinomas of the stomach and gastroesophageal junction show substantial genetic and spatial intratumoral heterogeneity.

**Copy number variation**

Next, we sought CNVs, i.e. homozygous or heterozygous deletions and amplifications. A total of 219 genes showed copy number variations, the majority of which were heterogeneous (Suppl. Table 13; Figure 3). The number of genes with CNVs per case ranged from 0 to 98 (Table 1). The highest number was found in case #5. Interestingly, this case did not have any obvious drivers in terms of SNVs and showed strong amplifications in MDM2, CD274 (PD-L1), JAK2, MYC, NOTCH2 as well as deletions in POLE1 and TGFBR2 in individual samples, which would point towards heterogeneity. Across all samples, we found examples for HER2 (validated independently by chromogenic in situ hybridization; case #3 and #4), MYC (case #5) and CDK12 (case #3 and #4) amplifications as well as CDKN2A (case #2, #3, #4 and #8), TP53 (case #3) and PTEN (Case #3 and #4) losses (Suppl. Table 13).

The homogeneous amplification of MDM2 and the heterogeneous amplification of CD274 (PD-L1) in case #5 were validated independently by fluorescence in situ hybridization (MDM2) and immunohistochemistry (Figure 3).

**Clonality analyses**

In view of the marked intratumoral heterogeneity we then assessed clonality and cancer cell fraction (CCF) on a per-sample basis and on a per-patient basis (Table 1). On a per sample basis, the percentage of clonal SNVs (non-synonymous or synonymous) ranged from 1 to 99% for non-synonymous mutations (Suppl. Table 6) and from 0 to 98.6% for synonymous mutations (data not shown). However, when clonality was assessed on a per-patient basis (all data for each patient were combined into a “single sample”), the number of clonal mutations ranged from 0 to 80 (median 1.0) for non-synonymous mutations and from 0 to 32 (median 1.0) for synonymous mutations (Table 1). These data show that a single sample analysis cannot reliably assess the clonality status of a somatic mutation and that the vast majority of the mutational landscape of GC is subclonal. However, the TP53 mutations in case #1, #2 and #3 were classified as clonal in the per-sample and in the per-patient analysis.

To further assess the reliability of the clonality estimations in our discovery cohort, we applied another approach, which was described by Opasic et al.\textsuperscript{13} and Werner et al.\textsuperscript{14} The number of samples required for correct identification of clonal mutations within the cancer with high certainty (>95%) was estimated. First, the “balance factor” $g$ was assessed for every individual tumor by fitting the information gain with each multi-region sample to a theoretical curve. Next, the information from the fully reconstructed multiregional trees and the branch-defining subclonal alterations were used to perform the estimation (Figure 4A). Six (case #1, #2, #3, #4, #8, #9) tumors were considered to be highly unbalanced, which implies that the number of “truly” clonal mutations was indeed low. The identification of these mutations would require the sequencing of many additional tumor samples. In one patient (case #6) with a fairly balanced phylogenetic tree ($g=0.56$), five samples were sufficient for the identification of clonal mutations with a probability >90%. For two other patients (case #5 and #7) with low estimated values of $g$ ($g=0.2$ and $g=0.01$) we could be fairly certain (>98% probability) that mutations from the root of the phylogenetic tree were indeed clonal using the existing number of samples (Figure 1).
Collectively, these data show that the assessment of clonality depends on the number of samples studied and the extent of intratumoral heterogeneity.

**Evolutionary trajectory**

Next, in order to investigate the variability in the mode of evolution in our discovery cohort we compared the variant allele frequency (VAF) histograms with the neutral model of cancer evolution as described initially by Williams et al.\(^\textsuperscript{11}\) using their neutralitytestr package (Figure 4B; Suppl. Figure 2).

To reduce the probability that apparent deviation from neutrality is caused by increase in allelic frequency due to gene duplication events, all variant alleles that had likely undergone gene doubling were removed, as shown by Williams et al.\(^\textsuperscript{15}\) All detected non-synonymous and synonymous mutations were included since a larger number of passenger mutations whose frequency had been increased by advantageous mutation supports the detection signs of selection.\(^\textsuperscript{16}\)

We conducted our analysis on VAFs without the purity correction, assuming that sample purity affects all variant frequencies equally. Therefore, a correction is unlikely to increase the resolution of our analysis. In addition, spatial constraints can introduce sampling bias into patterns of the clonal selection of the tumor,\(^\textsuperscript{17}\) therefore the analyses also included the average frequency of mutations from all available samples.

The VAF-histograms (Figure 4B; Suppl. Figure 2) show that the majority of VAF distributions found in individual samples is compatible with a neutral expectation, i.e. no selection. However, there is a high degree of heterogeneity in deviation from neutrality within each tumor, and VAF profiles collected from individual samples can lead to very different results for the evolutionary dynamics of cancer compared to the combination of all samples (Figure 4C).

This could be a result of distinct evolutionary trajectories within different parts of the tumor, or spatial effects of tumor growth and consequential sampling effects. Although we reject the neutral hypothesis in many cases, we do not observe clear subclonal peaks in our VAF-histograms that would imply strong selection in parts of the tumor.

**Pathway analysis including copy number variation**

Next, we assigned non-synonymous mutations and CNVs to pathways, which have been linked to GC, i.e. the SWI/SNF, TGF-β, Hippo, sonic hedgehog, NOTCH, WNT and JAK-STAT (Figure 2). The MSI GC showed the highest number of mutations (n=10) in a single pathway, while the remainder showed alterations in 1-5 genes per pathway (Suppl. Table 14). Again, a homogenous distribution of mutations was the exception and a heterogeneous distribution was the rule (Suppl. Table 14). Interestingly, homogeneous mutations occurred most commonly in the WNT signaling pathway (case #2, #3, #6, #8), the SWI/SNF pathway (case #3, #4, #6, #8) and the JAK/STAT-pathway (case #2, #5, #6). No homogenous mutation was found in the Notch pathway.

Recently, Park et al.\(^\textsuperscript{18}\) demonstrated in animal models that SMAD4 cooperates with p53 loss to promote the development and metastatic progression of GC. In support of the findings made in their animal model, our pathway analysis shows that four of five cases with \(TP53\) mutations (including all three cases with clonal \(TP53\) mutation) also had alterations in the TGFβ-pathway. Furthermore, \(SMAD4\) mutations and losses were only found in \(TP53\) mutant cases (Suppl. Table 14). In addition, all \(TP53\) mutant cases showed alterations in the SWI/SNF
pathway. He et al.\(^\text{19}\) provided evidence that members of the SWI/SNF pathway regulate cellular senescence via the p53/p21 and p16/pRB pathways.

Collectively, these findings lend support to the hypothesis that different pathways are effective during cancer development and progression, pointing towards interdependency: cancer progression in \(TP53\) mutant GCs is linked to subsequent (putative subclonal) alterations in the TGF\(\beta\) and SWI/SNF pathway.

**Phylogenetic analysis**

Next, we generated multiregional trees based on driver SNVs and CNVs. As shown in Figure 1 all tumors of our cohort provided evidence of branched somatic evolution, with the most complex being the MSI GC, confirming data published recently by von Loga et al.\(^\text{7}\) In addition, we inferred maximum parsimony trees in accordance with Lee et al.\(^\text{5}\) (Suppl. Figure 4). The authors observed a common phylogeny pattern of five cases with GC in which the primary genome is branched from a trunk while all the lymph node genomes (n=3 for each of the 5 cases) cluster in a separate branch. We did not observe this pattern for the single sample (case #5) for which lymph node data was available. In contrast to Lee et al.\(^\text{5}\), in case #5 the lymph node metastases did not cluster together in a separate, distinct branch, but rather clustered with different individual samples from the primary tumor (Figure 1). Comparing the multiregional trees with the maximum parsimony trees following the Lee et al.\(^\text{5}\) methodology, one can observe similar trees with differences attributed to methodology as well as the additional CNV data included in the multiregional trees. Generating LICHeE based multiregional trees including passenger mutation and/or synonymous mutations generated similar relationships in the trees (data not shown).

With regard to the TGF\(\beta\)-pathway, it was interesting to note that the \(SMAD4\) mutations of four cases with \(TP53\) mutations (i.e. cases #1, #2, #3, and #4) were subclonal and that different mutations of \(SMAD4\) aligned with different subclones providing circumstantial evidence for parallel somatic evolution (Figure 1).

**Decreased or lost expression of SMAD4 is associated with worse patient outcome**

Finally, we assessed the putative effect of SMAD4 alterations on patient prognosis. Using immunohistochemistry, we studied the expression of SMAD4 in case #1, #2, #3, and #4. Interestingly, SMAD4-immunostaining was heterogeneous and, occasionally, even a black-and-white staining pattern was noted, i.e. areas with complete loss of SMAD4 expression (both nuclear and cytoplasmic) were clearly demarcated from areas with retained expression (Figure 5).

Using a validation cohort (described in detail in Supplemental Materials and Methods), we aimed to test the hypothesis that a decreased or lost expression of SMAD4 would correlate with clinicopathological patient characteristics and patient outcomes. SMAD4 expression was studied using whole tissue sections and a validation cohort of 487 GCs (Figure 5; Suppl. Data 8; for further details see Supplemental Results).

A decreased expression of cytoplasmic SMAD4 (Q1-3 vs. Q4) correlated significantly with local tumor growth (\(p=0.003\); significant after correction for multiple testing) and UICC stage (\(p=0.007\); lost significance after multiple testing). No significant correlation was found between nuclear SMAD4 expression and any clinicopathological patient characteristic (Table 2).

The entire validation cohort showed a median overall survival (OS) of 14.1 months and a median tumor specific survival (TSS) of 15.5 months. Patient prognosis significantly depended on the Laurén-phenotype, T-, N-, M-, L-, V-,
Pn- and R-category, UICC-stage, lymph node ratio and cytoplasmic SMAD4 expression. Patients with SMAD4 loss showed significantly lower median overall survival (OS; 13.4 months, 95% C.I. 11.2 - 15.6; significant after multiple testing correction; p=0.001) and tumor specific survival (TSS; 14.9 months, 95% C.I. 12.1 - 17.7; significant after multiple testing correction; p=0.003) compared with retained SMAD4 expression (OS: 22.2 months, 95% C.I.10.0 - 34.5; TSS: 25.0 months, 95% C.I. 11.5 - 38.5)(Figure 5).

Discussion

The heterogeneity of malignant tumors is a major barrier to drug development and long-term disease control.\textsuperscript{20} It can be categorized into \textit{intertype heterogeneity} (differences between the cancers of two patients, each with a different tumor type), \textit{intratype heterogeneity} (cancers of the same type differ in two different individuals), \textit{intraprimary heterogeneity} (genetic heterogeneity between two cells of the same primary tumor), \textit{intermetastatic heterogeneity} (genetic heterogeneity between cells of different metastases) and \textit{intrametastatic heterogeneity} (genetic heterogeneity between two cells of the same metastasis).\textsuperscript{20} Intratype genetic heterogeneity lead to the proposal of four molecular subtypes of GC, i.e., EBV-positive, MSI-, CIN- and GS-GCs,\textsuperscript{4} each showing characteristic genetic alterations. However, comprehensive data on intraprimary and intermetastatic genetic heterogeneity in GC are scarce. Lee et al.\textsuperscript{5} performed whole-exome sequencing of 15 pairs of primary GC and their matched lymph node metastases in an Asian patient population and noted a substantial variation in the extent of mutational overlap or mutational heterogeneity between primary and lymph node metastasis genomes. Pectasides et al.\textsuperscript{6} analyzed patterns of heterogeneity in two independent patient cohorts. In the first cohort, only a single biopsy sample was obtained from the primary tumor of 11 patients and was compared with synchronous metastatic biopsies. In a second cohort, more than 100 samples were obtained from the primary tumors and metastatic sites of 26 patients and forwarded to targeted sequencing of a limited number of genes.\textsuperscript{6} They found discrepant pathogenic alterations between primary tumors and paired metastatic lesions in 45% of the patients. Among alterations in receptor tyrosine kinases, 9 of 12 cases (75%) were discordant across all matched samples.\textsuperscript{6} Von Loga et al.\textsuperscript{7} recently studied four MSI GCs by multiregional sequencing and found an extreme intratumoral heterogeneity as well as evidence of parallel evolution in this special subtype of GC. Likewise, in our cohort of nine GCs, the vast majority of non-synonymous mutations was distributed “heterogeneously” and not present in each patient sample. Even lymph node metastases showed heterogeneity among each other and in comparison with samples obtained from the primary tumor. Collectively, ours and other recent studies provide compelling evidence of a very complex intratumoral and intermetastasic genetic heterogeneity in GCs.

\textit{Inter-} and \textit{intratype} heterogeneity is related to environmental, patient and tissue-specific risk factors as well as poorly understood stochastic events leading to the malignant transformation of a hitherto non-neoplastic cell. This malignant transformation necessitates the deregulation of the diverse regulatory mechanisms of cell and tissue homeostasis, which have been summarized as the hallmarks of cancer.\textsuperscript{21} However, \textit{intraprimary} and \textit{intermetastatic} heterogeneity takes place after the malignant transformation of a single cell and originates from the evolutionary process, which already begins immediately after malignant transformation.\textsuperscript{22} Somatic evolution shapes the genetic diversity of a single tumor. This diversity is generated by mutation through selection and genetic drift, with additional influence from population structure and migration.\textsuperscript{22}

Multiregional sequencing studies provide evidence of somatic evolution shaping the genetic landscape of GC leading to a substantial \textit{intraprimary} and \textit{intermetastatic} heterogeneity.\textsuperscript{5, 6, 7} However, while heterogeneity may
shed some light on the subclonal architecture of the tumor, homogeneity does not necessarily represent clonality. Our bioinformatic approach demonstrates that only a minority of the homogeneously distributed mutations could be classified with reasonable certainty as clonal (Table 1) and that the assessment of clonality is a function of the existing mutational landscape (=interprimary heterogeneity) of the tumor and the number of samples available: in six cases, a correct assessment of clonality would require the analysis of additional samples for an accurate annotation, particularly in highly unbalanced tumors (Figure 4).

In malignant tumors, three modes of evolution may be operative: (1) in the “Big Bang” model, the tumor grows as a single “terminal” expansion populated by numerous heterogeneous subclones that are not subject to strong selection (=neutral expansion model); (2) the “sequential” or “linear” model describes clonal successions or sweeps that occur sequentially and (3) the “branched” evolution corresponds to a scenario where multiple subclonal alterations co-occur and compete during tumor growth.\textsuperscript{22} Using VAF histograms, we aimed to test the hypothesis that GCs follow the “Big Bang” model where all tumor-driving alterations responsible for cancer expansion appear to have been present in the first malignant cell.\textsuperscript{11, 23} Subsequent expansion is neutral and generates a large number of passenger mutations that are responsible for the extensive and common intratumoral heterogeneity.\textsuperscript{11, 23} Surprisingly, the compatibility with neutrality was variable between different samples from the same tumor contradicting the concept of a single mode of expansion for the entire tumor. This observation may also lend support to the hypothesis that different modes of evolution might be functional within a single tumor. However, it has to be kept in mind that even multiregional sequencing only provides a snap shot of a highly dynamic disease process and provides no information regarding the temporal and microenvironmental constraints. Intratumoral heterogeneity resulting from somatic evolution might be attributable to ongoing genetic and heritable epigenetic alterations and selection might be operative in certain but not all microenvironments, i.e. the different histological layers of the stomach wall, within lymph nodes or a metabolic environment mediated by chaotic angiogenesis, immune response, and various other factors.\textsuperscript{22}

Somatic evolution of GC has several major clinical implications regarding the assessment of putative subclonal genetic events as well as tissue-based precision medicine.

While clonal events might lead to cancer initiation, at later disease stages the initiating genetic lesions may no longer ensure cell survival or might have little influence on patient prognosis.\textsuperscript{22} By generating multiregional trees we identified subclonal SMAD4 mutations in four cases of the test cohort. Subsequent validation of the biological significance of the SMAD4 expression in a large Caucasian patient population provided strong evidence of the prognostic value of SMAD4 loss and correlated highly significantly with local tumor growth (T-category). It turned out to be an independent prognosticator of patient survival. Similar findings on the prognostic significance of SMAD4 in GC were made in three Asian cohorts\textsuperscript{24, 25, 26} and a single Caucasian cohort,\textsuperscript{27} however, none of these studies related their findings to intratumoral heterogeneity.

The subclonal alteration of SMAD4 (e.g. mutation and/or loss of heterozygosity), points towards another highly interesting issue: it was almost exclusively found in TP53 mutant GCs. Tumor initiating genetic events (=clonal ground state) may influence subsequent microevolutionary trajectories and may lead to parallel evolution, in which the fitness state of specific subclones depends on mutations in the same gene (SMAD4) or pathway (e.g. TGFb-signaling pathway). While these subclonal mutations could be missed by the analysis of a single bulk tissue sample (as shown here), identification of the clonal ground state may provide highly valuable information with regards to the future (most likely) subclonal alterations necessary for tumor progression. Tumor progression
could depend on epistatic genetic interactions in which the functional effects of genetic mutations are interdependent. Evolutionary trajectories might also partially explain the intratumoral heterogeneity: 382 genes harbored two to four different non-synonymous mutations in the same patient. Some might be irrelevant passenger mutations (according to the neutral model), but some might represent other evolutionary trajectories and the analysis of larger patient cohorts and in vivo disease models is urgently needed to identify further trajectories in GC. Each individual subcloncal mutation merits in-depth validation studies to explore its putative role in cancer biology since subclonal mutations could be clinically relevant as has been shown here concerning SMAD4.

With regard to TP53 mutant cases, we also noted subclonal alterations of members of the SWI/SNF pathway. This finding is in line with our recent study in which loss of ARID1A correlated inversely with MSI- and EBV status in GC, molecular subtypes of GC usually showing a low prevalence of TP53 mutations. Thus, alterations of the SWI/SNF pathway may point to an additional trajectory in TP53 mutant GCs. Parallel somatic evolution might also explain the intratumoral heterogeneity of PIK3CA mutations in EBV associated GCs. Thus, the subtle molecular subtypes of GC might be extended by the identification of subtype-specific evolutionary trajectories and further studies on this topic are urgently needed.

Intratumoral genetic heterogeneity carries a substantial risk for sampling errors when only a single tissue sample of the primary tumor or any metastasis is sequenced. This is most critical when druggable targets are sought such as HER2 or PD-L1. While intratumoral heterogeneity is a well-known feature of HER2, we provide evidence that this also could apply to PD-L1. A single case of our test cohort showed a heterogeneous amplification of CD274 (PD-L1). With regard to the assessment of the HER2-status, current recommendations state that, when possible, a minimum of five biopsy specimens, and optimally six to eight, should be obtained to account for intratumoral heterogeneity and to provide sufficient tumor specimens for diagnosis and biomarker testing. Likewise, it was shown in the past that the classification of clonal versus subclonal alterations depends on the number of samples studied. Furthermore, consideration of the spatial tumor structure decreases the probability to misclassify a subclonal mutation as clonal. All these data strongly argue against the application of companion diagnostics to a single tissue specimen or specimens obtained only from the primary tumor, although clinical reality often provides no choice. Alternatively, the identification and characterization of evolutionary trajectories may spare the search for subclonal mutations or decrease the risk of missing important mutations (e.g. SMAD4) once the “clonal ground stage” has been identified, which will inevitably lead to subclonal mutations relevant for tumor progression or disease control in the palliative setting. Thus, looking into the future of precision medicine, a combined approach using the identification of druggable targets by comprehensive molecular analysis, unveiling the mode of tumor expansion (neutral vs. non-neutral) and the discovery of evolutionary trajectories may aid in finding the best treatment for a particular tumor.

**Materials And Methods**

**Ethics statement**

Ethical approval was obtained from the local ethical review board (D 453/10 and D 525/15) of the University Hospital Schleswig-Holstein, Kiel, Germany.

**Study population and histology**
Discovery cohort (Table 1)

Between 2016 and 2017, we prospectively enrolled patients with an adenocarcinoma of the stomach or esophagogastric junction into the discovery cohort at the University Hospital Schleswig-Holstein, Campus Kiel. All patients were Caucasian patients from Northern Germany treated in a single center. The inclusion criteria were appropriate size of the primary tumor (diameter >3 cm) to enable multiregional tissue sampling without compromising the surgical pathological evaluation of the resection specimen. Immediately after the tumor was resected, the specimens were delivered on ice to the Department of Pathology. Depending on the size of the primary tumor, between 3 and 6 samples were punched out of the primary tumor using a core needle biopsy and frozen at -80°C until further use. Macroscopic pictures were taken from the surgical resection specimens before and after tissue sampling in order to facilitate anatomical reconstruction of the sampling procedure (Suppl. Figure 1).

Validation cohort

The validation cohort was collected from the archive of the Institute of Pathology, University Hospital Schleswig-Holstein, Campus Kiel. The cohort included all patients who had undergone either a total or partial gastrectomy for adenocarcinoma of the stomach or esophagogastric junction between 1997 and 2009. All tissue samples originated from routine therapeutic surgeries, for all of which the patients had given written informed consent. The following patient characteristics were retrieved: type of surgery, age at diagnosis, gender, tumor size, tumor localization, tumor type, depth of invasion, number of lymph nodes resected and number of lymph nodes with metastases. Patients were included if an adenocarcinoma of the stomach or esophagogastric junction was histologically confirmed. Exclusion criteria were defined as 1) histology identified a tumor type other than adenocarcinoma, and 2) patients had undergone perioperative or neoadjuvant chemo- or radiotherapy. Each resected specimen had undergone gross sectioning and histological examination by trained and board-certified surgical pathologists. For outcome analyses, the dates and causes of patients’ deaths were obtained from the Epidemiological Cancer Registry of the state of Schleswig-Holstein, Germany, thereby distinguishing between tumor-related deaths and deaths from other causes. Follow-up data of those patients who were still alive were retrieved from hospital records and general practitioners. All patient data were pseudonymized after study inclusion.

DNA sequence analysis (discovery cohort)

Genomic DNA was extracted from frozen tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Cryosections were prepared prior to DNA isolation to guarantee tumor cell content. DNA Exome libraries were prepared using the Nextera Rapid Capture Enrichment Kit, CEX version (Coding Exome Oligos; Illumina, San Diego, USA). Sequencing was performed on a Hiseq4000 instrument (Illumina) with 1% phiX (v3, Illumina) spike-in at 2*75 bp paired-end settings with the 150bp SBS chemistry.

DNA isolation from formalin-fixed and paraffin embedded tissue specimens

Genomic DNA was extracted from formalin-fixed and paraffin-embedded tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Tissue sections were manually microdissected prior to DNA isolation to ensure a tumor cell content of higher than 80%. The integrity and amplifiability of the isolated DNA was evaluated by a qualitative size range PCR assay.
Primary data analysis

Raw fastq data were quality-trimmed, and adapter sequences were removed using bbduk from the BBTools suite version 36.32 (http://sourceforge.net/projects/bbmap) with the following parameters: minlen=25 qtrim=r trimq=10 ktrim=r k=25 mink=11 hdist=1 overwrite=true tbo=t tpe=t. The Burrows-Wheeler aligner 0.7.15 (https://arxiv.org/abs/1303.3997) with default parameter settings was used to align the sequencing reads to the human reference genome (hs37d5). Duplicate reads were marked with sambamba 0.6.331 and indel realignment was performed using ABRA version 0.9732 FastQC 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Qualimap 2.233 were used to perform quality checks on the fastq and bam files respectively.

Somatic mutation calling

Paired tumor-normal variant calling was performed using VarDict 1.5.134 with the following parameters: mapping quality Q=10, base quality phred score q=20, minimum allele frequency f=0.01, and number of nucleotides to extend for each segment x=2000. Additionally, the read position filter P=0.9 and maximum number of reads with mismatches m=4.25, were supplied to var2vcf_paired.pl with further downstream filtering steps to improve detection of low frequency variants as described by Brad Chapman (http://bcb.io/2016/04/04/vardict-filtering/). ANNOVAR35 was used to annotate variants utilizing refGene, cosmic84, clinvar_20170905, icgc21, nci60, exac03, exac03nontcga, snp142, avsnp150, 1000g2015aug_all, lj26_all, dbnsfp33a, and intervar_20180118 databases. Homopolymer regions were marked using vcfpolyx from the jvarkit suite (https://github.com/lindenb/jvarkit). Variants were retained if the following criteria were met: allele frequency (AF)≥5%, total read depth ≥10 in either the tumor or the normal sample, variant depth in tumor sample ≥2, no strand bias, AF>10% in homopolymer regions. Variants in blacklisted regions described by Fuentes et al.36 as well as ENCODE37 were filtered out. In addition, annotation-based filtering was utilized to retain only coding, non-synonymous variants with ExAC AF<0.01.38 Variants unknown to either COSMIC or ICGC were retained only if they occurred within one of the 719 genes of the COSMIC Cancer Gene Census (https://cancer.sanger.ac.uk/census, downloaded June 12, 2018)39(https://dcc.icgc.org/). An additional variant recovery process on the list of filtered variants was implemented on a per patient basis to recover variants from individual samples with AF<5% if they were present in at least one other sample of the same patient with AF≥5%. Tumor purity and ploidy were computationally estimated using Sequenza.40 Clonality and cancer cell fraction (CCF) for each variant was determined using Palimpsest.41 In brief, CCF is computed by adjusting the variant allele fraction for the tumor purity and the absolute copy number at each locus in tumor and normal cells. Mutations were classified as subclonal if the upper boundary of the 95% confidence interval was below the threshold of 0.95. COSMIC Mutational Signatures Version 2 were inferred using the R package deconstructSigs.42

Copy number profiling

Allele-specific copy number calling was done using CNVkit 0.9.5.43 A pooled normal reference was created from the nine matched non-neoplastic stomach mucosa samples. The initial segments were called with a conservative significance threshold of t = 1e-6 and low coverage segments were dropped. Segments were then used along with raw variant calls from VarDict, the estimated tumor purity, to call major and minor copy number variants and annotated by ANNOVAR based on the refGene database. Calls were considered as deletions when total copy number was 0, and as amplifications when total copy number was at least 6.
**Tumor mutation burden, microsatellite instability and viral sequence analysis**

Tumor mutation burden was calculated for each sample in terms of the number of non-synonymous variants per 1 Mb and scaled according to the exome panel size. Microsatellite instability (MSI) status was determined by MSIsensor\(^4^4\) with a threshold of <10% for MSS (microsatellite stable), <10% and >30% for MSI-L (low), and >30% for MSI-H (high). To screen for viral integration events, unmapped reads were aligned against a sequence database of 198 human viruses (EBV, human papilloma virus, herpes simplex virus, among others).

**Phylogenetic analysis**

Patient-specific multiregional trees from CCF data were constructed using LICHeE.\(^4^5\) Since LICHeE is limited to constructing trees based on single nucleotide variations (SNV) only, copy number variants (CNV) were manually incorporated into the SNV-based trees. Driver CNVs as identified by Cancer Genome Interpreter, were added to the SNV-based trees; for some patients this did not result in any changes to the tree nodes, whereas for some patients the internal tree nodes were redrawn to reflect the additional CNVs. In addition, we constructed maximum parsimony trees based on a binary matrix of SNVs per patient using a branch-and-bound algorithm with PHYLIP (Felsenstein, J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle).

**Validation analyses using Sanger sequencing, pyrosequencing and digital polymerase chain reaction**

In order to validate of the heterogeneous mutational patterns, Sanger sequencing analysis of *ASXL3*, *TP53* (exon 5 and 8) and *SMAD4* (exon 2, 9 and 11) was done using the PyroMark PCR Kit (Qiagen). PCR products were purified using the NucleoSpin® Gel and PCR Clean-up (Machery-Nagel, Düren, Germany) and sequenced by dye terminator cycle sequencing (BigDye Terminator v1.1 Cycle Sequencing kit, Applied Biosystems, Darmstadt, Germany) with universal M13- or PCR Primers. The sequencing products were purified using the DyeEx 96 Kit (Qiagen) and analyzed on a Genetic Analyzer 3500 (Applied Biosystems). Pyrosequencing, using the PyroMark PCR Kit (Qiagen) and the PyroMark Gold Q24 Reagents (Qiagen) were done to detect SNPs in *BRCA1*, *BRCA2*, *CDH1*, *CTNNB1*, *KRAS*, *MLH1*, *MUTYH*, *PIK3CA*, *POLE*, and *RNF43*. The PyroMark Q24 System and PyroMark analysis software (both Qiagen) were used for analysis. To validate low frequent mutations in *ARID1A*, *ARID1B*, *AKT1*, *CLOCK*, *FLT4*, *IKKB*, *IKZF3*, *LRP1B*, *MAP2K4*, *MCM8*, *PAX5*, *PRRC2A*, and *TP53BP* digital PCR were done using the ddPCR™ Supermix for Probes (No dUTP) and the QX200™ Droplet Digital™ PCR System (both Biorad) following the manufacturer's instructions. The primer sequences used are listed in Suppl. Table 1. Suppl. Table 2 summarizes the validated mutations.

**Histology**

Tissue specimens used for histology and immunohistochemistry were fixed in formalin and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin. Histological examination of primary tissue sections was carried out for all cases (discovery and validation cohort) to assure if inclusion criteria were met. Tumors were classified according to the Laurén classification.\(^4^6\) pTNM-stage of all study patients was determined according to the eighth edition of the UICC guidelines.\(^4^7\)

**Immunohistochemistry, scoring of SMAD4-immunostaining and virtual microscopy**
Immunohistochemistry was carried out with antibodies directed against SMAD4 (dilution 1:50; monoclonal rabbit; 50, Cell Signaling Technology Europe, Frankfurt am Main, Germany) and PD-L1 (dilution 1:100, E1L3N, Cell Signaling Technology) using whole tissue sections. Immunostaining was performed with the autostainer Bond™ Max System (Leica Microsystems GmbH, Wetzlar, Germany). The immunoreaction was visualized with the Bond™ Polymer Refine Detection Kit (brown labelling; Novocastra; Leica Microsystems, Wetzlar, Germany).

Scoring of each tumor was assessed by determining a histoscore (H-score), following a semi-quantitative approach combining both the immunostaining intensities (subsequently referred to as IHC-scores) and the percentages of positive cells of the tumor. The IHC-score was based on tumor cells showing either strong (3+), intermediate (2+), weak (1+) or no (0) staining of SMAD4 in the cytoplasm and nucleus, respectively. Tumor cells without detectable cytoplasmic or nuclear staining were scored with 0. The percentage of positive tumor cells (approximated to the nearest 10) showing the defined staining intensities (3+, 2+, 1+, 0) was gauged with respect to all tumor cells visible on each tissue specimen and always added up to a total of 100% tumor cells. Finally, an H-score was calculated according to the formula: H-score = [0 x percentage of immunonegative tumor cells] + [1 x percentage of weakly stained tumor cells] + [2 x percentage of intermediately stained tumor cells] + [3 x percentage of strongly stained tumor cells]. The maximum possible H-score was 300, if all cells of a given tumor sample showed a strong staining: [0 x 0%] + [1 x 0%] + [2 x 0%] + [3 x 100%] = 300. The multipliers within the formula yielded an improved stratification of the H-scores: tumor samples with a predominantly high staining intensity and such samples with a predominantly low staining intensity were more distinctly separated.

For virtual microscopy with area analysis for tumor heterogeneity, tissue slides were scanned using a Leica SCN400 microscopic scanner (Leica Biosystems, Nussloch, Germany).

**MDM2 fluorescence in situ hybridization**

Analysis of MDM2 amplification was done by fluorescence in situ hybridization using the Vysis MDM2/CEP 12 FISH Probe Kit (Abbott Diagnostika MediSense, Wiesbaden, Germany) following standard procedures. The results of FISH were evaluated by screening the entire tissue sections. Subsequently, MDM2 and centromer 12 signals were counted in at least 20 representative adjacent cancer cell nuclei within the invasive region. The presence of FISH clusters was noted and the ratio of MDM2/centromer 12 signals was calculated. The gene count was calculated by dividing the number of MDM2 gene signals by the number of cancer cell nuclei studied.

**Assessment of further clinicopathological characteristics**

*H. pylori*,

Epstein-Barr virus,

microsatellite (MSI),

and HER2 status were assessed as described previously.

**Statistical methods**

SPSS version 24.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. The correlation between non-ordinal clinicopathological patient characteristics and SMAD4 was tested with Fisher’s exact test. T category, N category, UICC stage and tumor grading as variables of ordinal scale were tested with Kendall’s tau-test. Median survival with 95% confidence intervals was determined by the Kaplan-Meier method. Differences between median survivals were tested with the log-rank test. A multivariate survival analysis (Cox regression) was performed. A p-value of ≤0.05 was considered to be significant. All p-values are given uncorrected. The Siemes (Benjamini-Hochberg) procedure was applied to compensate for false discovery rate. Any P-values that lost significance are marked.
Abbreviations

AF - allele frequency; CIN - chromosomal instability; CNV – copy number variation; EBV - Epstein-Barr virus; GC - gastric cancer; GS - genomic stability; MSI - microsatellite instability; SNV - Single nucleotide variations; VAF - variant allele frequency

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the local ethical review board (D 453/10 and D 525/15) of the University Hospital Schleswig-Holstein, Kiel, Germany.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]. Raw sequencing data are available at the European Genome Archive (EGAS00001004525).

Competing interests

The authors declare no conflict of interest

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Authors' contributions

Study concept and design was done by CR, CH, PR, SK and TM. Surgical pathological data were acquired by CR, SK, CH and JHE. Tissue specimens and clinical data were provided by JHE. Validation studies were done by SK, AL, CH and JH. The data were analyzed and interpreted by AA, CR, SK, LO, AT, TM. Drafting of the manuscript and critical revision of the manuscript for important intellectual content was done by all authors. Administrative, technical, or material support was provided by CR, JHE and PR. The study was supervised by CR, PR and TM.

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Tables

Table 1: Clinicopathological patient characteristics of the gastric cancer test cohort
| Case | Case #1 | Case #2 | Case #3 | Case #4 | Case #5 | Case #6 | Case #7 | Case #8 | Case #9 |
|------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Gender | m | m | m | m | m | m | m | m | m |
| Age | 62 | 68 | 70 | 85 | 50 | 80 | 56 | 77 | 63 |
| Localization | Cardia | Cardia | Antrum/Corpus | Antrum | Fundus | Cardia | Cardia | Cardia | Antrum |
| Neoadjuvant treatment | yes | yes | no | no | no | no | yes | no | yes |
| Laurén phenotype | intestinal | mixed | mixed | mixed | diffuse | intestinal | intestinal | mixed | diffuse |
| Tumor size (cm) | 3.8 | 4.4 | 12.9 | 4.0 | 4.8 | 4.1 | 6.7 | 6.2 | 3.7 |
| Epstein Barr virus status | negative | negative | negative | negative | negative | negative | negative | negative | negative |
| Lauren phenotype | intestinal | mixed | mixed | mixed | diffuse | intestinal | intestinal | mixed | diffuse |
| Tumor size (cm) | 3.8 | 4.4 | 12.9 | 4.0 | 4.8 | 4.1 | 6.7 | 6.2 | 3.7 |
| Epstein Barr virus status | negative | negative | negative | negative | negative | negative | negative | negative | negative |
| Lauren phenotype | intestinal | mixed | mixed | mixed | diffuse | intestinal | intestinal | mixed | diffuse |
| Number of samples sequenced | 4 | 3 | 6 | 5 | 10 | 5 | 4 | 6 | 5 |
| Non-synonymous mutations (total valid) | 184 | 373 | 714 | 348 | 425 | 3111 | 181 | 369 | 242 |
| Copy number variations (number of genes) | 6 | 22 | 65 | 11 | 98 | 3 | 5 | 1 | 0 |
| Tumor sample | Number of samples with same nonsynonymous mutation | |
| 1 sample | 71 (38.6%) | 150 (42.6%) | 350 (49.0%) | 133 (38.2%) | 211 (49.6%) | 1262 (40.6%) | 112 (61.9%) | 235 (63.7%) | 140 (57.8%) |
| 2 samples | 25 (13.6%) | 59 (15.8%) | 78 (10.9%) | 15 (4.3%) | 29 (6.8%) | 360 (11.6%) | 24 (13.3%) | 25 (6.8%) | 30 (12.4%) |
| 3 samples | 58 (31.5%) | 155 (41.6%) | 31 (4.3%) | 11 (3.2%) | 27 (6.4%) | 180 (5.8%) | 18 (9.9%) | 9 (2.4%) | 23 (9.5%) |
| 4 samples | 30 (16.3%) | 8 (1.1%) | 26 (7.5%) | 17 (4.0%) | 148 (4.8%) | 27 (14.9%) | 14 (3.8%) | 28 (11.6%) | 21 (8.7%) |
| 5 samples | 25 (3.5%) | 163 (46.8%) | 13 (3.1%) | 1161 (37.3%) | 17 (4.6%) | 21 (8.7%) |
| 6 samples | 222 (31.1%) | 18 (4.2%) | 18 (4.2%) | 69 (18.7%) | |
| 7 samples | 24 (5.6%) | |
| 8 samples | 15 (3.5%) | |
| 9 samples | 25 (5.9%) | |
| 10 samples | 46 (10.8%) | |
| Per patient Clonality | |
| Clonal (non-synonymous) | 2 (2.0%) | 80 (50.0%) | 62 (23.9%) | 1 (0.9%) | 1 (1.3%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Subclonal (non-synonymous) | 98 (98.0%) | 80 (50.0%) | 197 (76.1%) | 110 (98.2%) | 76 (98.7%) | 1083 (100%) | 48 (100%) | 81 (100%) | 106 (100%) |
| Not assessable (non-synonymous) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (0.9%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
### Table 2 SMAD4 expression in the validation cohort and correlation with clinicopathological patient characteristics

| Total (non-synonymous) | 100 | 160 | 259 | 112 | 77 | 1083 | 48 | 81 | 106 |
|------------------------|-----|-----|-----|-----|----|------|----|----|-----|
| Clonal (synonymous)    | 1   | 32  | 26  | 2   | 1  | 0    | 1  | 0  | 0   |
|                        | (6.0%) | (15.9%) | (10.1%) | (1.3%) | (5.7%) | (0%) | (0.4%) | (0%) | (0%) |
| Subclonal (synonymous) | 159 | 168 | 231 | 157 | 174| 825  | 229| 168| 257 |
|                        | (94.0%) | (83.6%) | (89.9%) | (98.7%) | (99.4%) | (97.6%) | (99.6%) | (100%) | (99.6%) |
| Not assessable (synonymous) | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 1 |
|                        | (0%) | (4.9%) | (0%) | (0%) | (0%) | (2.4%) | (0%) | (0%) | (0.4%) |
| Total (synonymous)     | 160 | 201 | 257 | 159 | 175| 827  | 230| 168| 258 |
|                     | SMAD4 cytoplasmatic expression | SMAD4 nuclear expression |
|---------------------|---------------------------------|--------------------------|
|                     | Total valid                     | HScore 0 present         | HScore 0 absent      |
|                     | n (%)                           | n (%)                    | p-value               |
| Total               | 385 (79.1)                      | 102 (20.9)               | 279 (57.29)           |
| Gender              | 182 (37.4)                      | 147 (80.8)               | 93 (51.1)             |
|                     | Male 305 (62.6)                 | 238 (78.0)               | 186 (61.0)            |
|                     | Age Group                       |                           |                        |
|                     | < 68 Years 238 (49.5)           | 195 (81.9)               | 137 (57.6)            |
|                     | ≥68 Years 243 (50.5)            | 185 (76.1)               | 139 (57.2)            |
| Localization        | Proximal stomach 147 (30.9)     | 125 (85.0)               | 95 (64.6)             |
|                     | Distal stomach 328 (69.1)       | 251 (76.5)               | 177 (50.4)            |
| Laurén phenotype    | Intestinal 254 (52.2)           | 199 (78.3)               | 160 (63.0)            |
|                     | Diffuse 151 (31.0)              | 125 (82.8)               | 76 (50.3)             |
|                     | Mixed 31 (6.4)                  | 24 (77.4)                | 19 (61.3)             |
|                     | Unclassifiable 51 (10.5)        | 37 (72.5)                | 24 (71.4)             |
| Grading             | G1 / G2 116 (24.0)              | 87 (75.0)                | 68 (58.6)             |
|                     | G3 / G4 367 (76.0)              | 294 (80.1)               | 210 (57.2)            |
| pT category         | T1a / T1b 61 (12.6)             | 38 (62.3)                | 34 (55.7)             |
|                     | T2 56 (11.5)                    | 41 (73.2)                | 30 (53.6)             |
|                     | T3 193 (39.7)                   | 160 (82.9)               | 116 (60.1)            |
|                     | T4a / T4b 176 (36.2)            | 146 (83.0)               | 98 (55.7)             |
| pN category         | N0 138 (28.6)                   | 103 (74.6)               | 73 (52.9)             |
|                     | N1 69 (14.3)                    | 52 (75.4)                | 44 (63.8)             |
|                     | N2 87 (18.0)                    | 72 (82.8)                | 51 (58.6)             |
|                     | N3a/b 189 (39.1)                | 154 (81.5)               | 110 (58.2)            |
| M category          | M0 395 (81.1)                   | 306 (77.5)               | 229 (58.0)            |
|                     | M1 92 (18.9)                    | 79 (85.9)                | 50 (54.3)             |
| UICC stage          | IA / IB 81 (16.8)               | 56 (69.1)                | 42 (51.9)             |
|                     | IIA / IIB 107 (22.2)            | 83 (77.6)                | 65 (60.7)             |
|                     | IIIA / IIB / IIEC 203 (42.0)    | 164 (80.8)               | 121 (59.6)            |
|                     | IV 92 (19.0)                    | 79 (85.9)                | 50 (54.3)             |
| Lymph node ratio    | Low (<0.189)                    | 182 (77.1)               | 133 (56.4)            |
|                     | High (≥0.189)                   | 199 (80.6)               | 145 (58.7)            |
| pL category         | L0 219 (48.5)                   | 170 (77.6)               | 115 (52.5)            |
|                     | L1 233 (51.5)                   | 185 (79.4)               | 142 (60.9)            |
| pV category         | V0 400 (88.7)                   | 315 (78.8)               | 231 (57.8)            |
|                     | V1 51 (11.3)                    | 39 (76.5)                | 26 (51.0)             |
| R status            | R0 408 (87.2)                   | 318 (77.9)               | 236 (57.8)            |
|                     | R1 / R2 60 (12.8)               | 52 (86.7)                | 32 (53.3)             |
| HER2 status         | Negative 406 (92.1)             | 328 (80.8)               | 234 (57.6)            |
|                     | Positive 35 (7.9)               | 27 (77.1)                | 20 (57.1)             |
| H. pylori status    | Negative 346 (85.0)             | 273 (78.9)               | 200 (57.8)            |
|                     | Positive 61 (15.0)              | 42 (68.9)                | 30 (49.2)             |
| EBV status          | Negative 449 (95.3)             | 358 (79.7)               | 255 (56.8)            |
|                     | Positive 22 (4.7)               | 16 (72.7)                | 14 (63.6)             |
| MSI status          | MSS 435 (92.6)                  | 351 (80.7)               | 251 (57.7)            |
|                     | MSI 35 (7.4)                    | 21 (60.0)                | 17 (48.6)             |
| Overall             | Total / events / censored 467   | 370 / 301 / 69.97       | 269 / 221 / 48.19    |
| Survival            | Median Survival 13.4 ± 1.1      | 22.2 ± 6.3               | 14.7 ± 1.3            |
|                     | 95% C.I. 11.2 - 15.6            | 10.0 - 34.5              | 12.0 - 17.3           |
| Tumor Specific      | Total / events / censored 439   | 346 / 247 / 99.93       | 252 / 183 / 69.18    |
| Survival            | Median Survival 14.9 ± 1.4      | 25.0 ± 6.9               | 15.5 ± 1.6            |
Figures

Figure 1

Discovery cohort and multiregional trees Schematic representation of the nine patients from the discovery cohort. Multiregional trees provide evidence of somatic evolution. Text in green and red indicate amplifications and deletions; italics represent predicted drivers, while others are known drivers as determined by Cancer Genome Interpreter. Variants denoted by an asterisk are those that are present in more than one branch of a tree, and could not be satisfactorily resolved into a single branch.
Figure 2

Intratumoral heterogeneity and pathway analysis (A) Non-synonymous mutations were unevenly distributed among patients and patient samples. Each row represents a patient sample and each column represents one non-synonymous mutation. (B) Assignment of mutations to pathways, i.e. the SWI/SNF, TGF-beta, Hippo, sonic hedgehog, NOTCH, WNT and JAK-STAT pathway, also showed marked intratumoral heterogeneity and provided evidence of parallel evolution.
Figure 3

Copy number variation (A) Copy number variation analyses showed marked intratumoral heterogeneity (maroon denotes amplification and dark blue deletion). (B-I) Case #5 yields homogeneous amplifications in MDM2 (all ten samples) and a heterogeneous amplification of CD274 (PD-L1; 2/10 samples including a single lymph node metastasis). MDM2-amplification was confirmed independently in all samples, i.e. primary tumor (B & E; nonneoplastic mucosa as a reference in C) and all lymph node metastases (D). Amplification of CD274 was associated with strong PD-L1 immunostaining only in a single sample (H) and only in a single lymph node metastasis. All other samples were immunonegative for PD-L1 (I). The PD-L1-positive tumor area (E) showed a phenotype, different from the remainder (G). Primary tumor (B); corresponding nonneoplastic mucosa (C); lymph node metastasis corresponding to sample G13390 (D, F, H) and a sample of the primary tumor without CD274 amplification (PD-L1-immunonegative; E, G, I). Fluorescence in situ-hybridization (orange signal: MDM2, green signal: reference centromere; B-E); H&E- staining (F; G) and anti-PD-L1-immunostaining (H, I). Original magnifications 1000-fold (B-E), 400-fold (F-I).

Figure 4

Clonality and neutrality in the discovery cohort (A) Clonality was assessed as described. Case #1, #2, #3, #4, #8, and #9 are highly unbalanced and additional samples would be needed for correct estimation of clonality. In three cases (case #5, #6, and #7) we could be fairly certain that mutations from the root of the phylogenetic tree were indeed clonal using the existing number of samples. (B, C) The neutral model assumes that there are no selective differences, such that the number of mutations of a certain allelic frequency declines as the inverse of that frequency. Here, we show the agreement between each tumor sample and this neutral expectation. (B) Illustrates neutrality analysis of the samples from case #5. Left column: variant allele frequency histogram. Dark grey shade marks interval used for comparison with the neutral model. Central column: shows increment in the cumulative number of mutation with inverse allelic frequency 1/f (black dots) and linear model best fit (red line). Light grey marks samples that are in agreement with the neutral model R2 ≥ 0.98. Right column: normalized cumulative distribution of mutations and theoretical model. Distance between distributions was quantified using a Kolmogorov-Smirnov test. (C) Summarizes neutrality analyses for cases #1 to #5, #7 to #9. Case #6 (MSI) was not included in the neutrality analysis as a large, likely clonal, peak covered the most of the frequency range obfuscating the distribution of subclonal mutations. The agreement is quantified by the Kolmogorov-Smirnov test, where the Kolmogorov distance between the empirical and the theoretical distribution is shown for each
sample. The normalized cumulative number of putatively subclonal mutations in a frequency area below the clonal peak was used where a power-law distributed subclonal tail of mutations would be expected in the model of neutral evolution. The lines represent the standard deviation of the Kolmogorov distance across samples per patient.

**Figure 5**

SMAD4 is heterogeneously expressed in gastric cancer and a decreased expression correlates with patient survival (validation cohort) References for immunostaining analysis according to H-score. Staining intensities ranged from 0 (A; nuclear and cytoplasmic negative) to 3+ (D, nuclear and cytoplasmic strong expression) with 1+ (B; nuclear and cytoplasmic weak expression) and 2+ (C; nuclear and cytoplasmic moderate expression) in between. Black-and-white expression of SMAD4 describes tumors with clearly demarcated areas of complete loss of nuclear and cytoplasmic SMAD4 expression next to areas with retained expression (E). Translation of genetic heterogeneity into intratumoral phenotypic heterogeneity of SMAD4 expression is further exemplified in (F): Tissue sections obtained from three separate paraffin blocks of the primary tumor of case #3 were stained with an antibody directed against SMAD4. Subsequently the viewer and painting program VMP was used to mark the tumor compartments with strong cytoplasmic SMAD4 expression (3+; orange color) and decreased SMAD4 expression (0, 1+, 2+); blue color. Anti-SMAD4 immunostaining, hematoxylin counterstain; 400 x (A-D) and 100 x (E) magnifications. (G) Kaplan-Meier curves depicting patients’ survival according to SMAD4 status (Q1-3 vs. Q4; for further details see Suppl. Results). Kaplan-Meier curves demonstrating correlations between cytoplasmic SMAD4 (top row) and nuclear (bottom row) loss in tumor cells and overall as well as tumor-specific survival.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- RockenetalSupplMethodsandSupplFigures.pdf
• RockenetalSupplTable1Primersequencesfinal.xlsx
• RockenetalSupplTable2Validationfinal.xlsx
• RockenetalSupplTable3SequencingStatisticsfinal.xlsx
• RockenetalSupplTable4VARs nonsynonymous final.xlsx
• RockenetalSupplTable5VARs synonymous final.xlsx
• RockenetalSupplTable6CCF final.xlsx
• RockenetalSupplTable7purity ploidy final.xlsx
• RockenetalSupplTable8homo and heterogeneous final.xlsx
• RockenetalSupplTable9multiple mutations final.xlsx
• RockenetalSupplTable10primary vs lymphnode final.xlsx
• RockenetalSupplTable11Comparison final.xlsx
• RockenetalSupplTable12mutations and more final.xlsx
• RockenetalSupplTable13CNV final.xlsx
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