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Molecular Classification of Resected Primary Duodenal Adenocarcinoma

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ABSTRACT

Introduction: The molecular and histological characteristics of primary duodenal adenocarcinoma (DA) have been poorly described, which hampers the development of new treatment options. This study aimed to characterize the landscape of chromosomal copy number aberrations (CNAs), microsatellite instability status, and tumor–stroma content, and their association with clinicopathological characteristics of patients with DA.

Methods: DNA was extracted from tumor tissues of patients who underwent a primary surgical resection for DA in a single center (2000–2019). Shallow whole genome sequencing (sWGS) was performed to identify chromosomal CNAs and the genomic instability index (GII), for which 25% of the genome was altered, was used to classify tumors as CNA^{low} or CNA^{high}. A PCR-based assay was performed to classify tumors as microsatellite stable (MSS) or instable (MSI). Immunohistochemistry and digital image analysis were performed to determine the tumor–stroma content, for which 50% stroma content was used to classify tumors as $stroma^{low}$ or $stroma^{high}$.

Results: Among 74 patients with resected DA, sWGS identified 39 (52.7%) CNA^{low} and 35 (47.3%) CNA^{high} tumors. Overall, 16 (21.6%) DAs were MSI. All MSI tumors were CNA^{low}. The tumor–stroma content was low in 51 (68.9%) and high in 23 (31.1%) of DAs. CNA status was most predictive for 5-year overall survival: 45.5% for patients with CNA^{low} compared to 31.0% for patients with CNA^{high} DA (HR 2.20, 95% CI 1.12–4.30, $p = 0.02$).

Conclusion: About half of resected DAs had CNA^{low}, which was associated with the most favorable prognosis. Subgrouping of DA could be used for patient stratification in future trials testing novel therapies.

Jacob de Bakker and Hedde Biesma share the first authorship.

Nicole van Grieken and Geert Kazemier share the senior authorship.

1 | Introduction

Primary duodenal adenocarcinoma (DA) is the most common malignant neoplasm of the small bowel. [1, 2] However, little is known about its molecular and histopathological characteristics and their respective clinical behavior. Over the past 10 years, substantial efforts have been made to investigate the molecular characteristics of gastrointestinal (GI) tract cancers, such as The Cancer Genome Atlas (TCGA) classification for gastric cancer (GC) and the Consensus Molecular Subtypes (CMS) for colorectal cancer (CRC), respectively [3, 4]. Gastric adenocarcinomas are classified by TCGA into Epstein–Barr virus positive (EBV+), microsatellite instable (MSI), genomically stable (GS), and chromosomal instable (CIN) subtypes. The latter two were defined based on the abundance of specific copy number aberrations (CNAs) in the genome [3]. Best survival was seen for patients with EBV+ or MSI tumors compared to GS and CIN GC [5, 6]. In CRC, the CMS taxonomy distinguished four subtypes by their gene expression profiles: CMS1 is characterized by hypermutated and MSI tumors, CMS2 consists largely of canonical epithelial tumors, CMS3 features frequent *KRAS* mutations and metabolic dysregulation, and CMS4 cancers are characterized by stromal invasion and angiogenesis [4]. Worst survival was observed for patients with CMS4 tumors in local disease, and those with CMS1 in advanced stages of CRC. These subtypes of GC and CRC exhibit fundamental differences in histopathological, molecular, and clinical characteristics [5, 7].

Here, we investigated the histopathological and molecular makeup of resected DA and their correlation with clinical characteristics. CNAs as well as MSI status were analyzed, since these factors play a major role in the TCGA subtypes of GC and the CMSs of CRC. Tumor–stroma content was also investigated, since this is an important feature of CMS4 CRCs and other mesenchymal GI subtypes. Correlative analyses could lead to a better understanding of the biology of DA, which could eventually guide precision medicine and improve patient outcomes.

2 | Materials and Methods

2.1 | Patients

This is a single-center retrospective study including consecutive patients undergoing resection of DA between 2000 and 2019 at Amsterdam UMC, a tertiary referral center. Patient files were screened for their clinicopathological features. All patients were included independently of stage of disease. Representative hematoxylin and eosin (H&E) slides from each specimen were selected by a GI pathologist (NCTvG) for tumor content. Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were collected from the archives of the Department of Pathology.

All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. Written informed consent for molecular analyses was previously obtained from all patients.

2.2 | DNA Isolation and MSI Analysis

Tumor DNA was extracted as previously described [8]. Briefly, serial sections of 10 μm were cut and mounted on glass slides. Tumor areas were annotated by a dedicated GI pathologist (NCTvG) on H&E-stained slides and macrodissected with a scalpel followed by genomic DNA extraction (QIAamp DNA Micro kit, Qiagen, Westburg, Leusden, the Netherlands).

Tumor DNA was analyzed for MSI status as previously described [5]. Briefly, five near-monomorphic mononucleotide markers (BAT-25, BAT-26, MONO-27, NR-21, and NR-24) were assessed using a fluorescent multiplex PCR-based method (MSI Analysis System, Promega Corporation, Madison, WI, USA). Tumors were considered MSI if at least two out of five mononucleotide markers showed instability, and as MSS if one or none of the markers showed instability. Immunohistochemistry (IHC) for mismatch repair proteins (MLH-1, MSH-2, MSH-6, PMS-2) was performed on one tumor sample after MSI-PCR failed. Tumors were considered MSI if one of these four proteins was not expressed by IHC.

2.3 | Genome-Wide CNA Analysis

To determine CNAs, DNA libraries for shallow whole genome sequencing were prepared using TruSeq Nano kits (Illumina, San Diego, CA, USA). Libraries were sequenced on a HiSeq4000 or NextSeq2000 (Illumina). Data analysis was performed in R (version 3.6.1). R package QDNAseq (version 1.28.0) was used to extract CNAs to count the number of sequence reads per 100 kbp bin, followed by masking and corrections of sequencing biases [9]. The CNA profiles were dewaved by the R package NoWaves (version 0.6) [10], and segmented by the R package DNAcopy (version 1.66.0) [11]. Tumor cell percentages were estimated using the R package ACE (version 1.10.0) with default settings and a penalty of 0.5 [12]. Finally, CNA calls were made by the R package CGHcall (version 2.54.0) [13] with the tumor percentage as derived from ACE as input variable for the cellularity of each tumor. Since a very low tumor percentage derived by ACE could lead to an overcorrection of CNA calls, the tumor percentage by ACE closest to the tumor percentage estimation by the pathologist was selected. Frequency plots of CNAs were made with R package CGHregions (version 1.40.0) [14].

The percentage of the genome that was altered was calculated using CNAs. Called CNA data of each segment was divided into: −2 (deep deletion), −1 (loss), 0 (no CNA), +1 (gain), and +2 (amplification). The length of the segments with a called CNA (i.e., CNA calls −2, −1, 1, and 2) was combined for each sample and divided by the total length of the genome that could be called and multiplied by 100 to calculate the percentage of the genome that was altered, also known as the genomic instability index (GII). A cutoff of 25% was used to determine CNA^{low} versus CNA^{high}, as previously applied for CRC [15]. Hence, a tumor is considered CNA^{high} if at least 25% of the length of the genome harbors a called CNA.

2.4 | Immunohistochemistry

Tissue sections of 4 μm thickness were cut and mounted onto positively charged glass slides (TOMO, Matsunami Glass, Ind., Osaka,

Japan) and heated at 60°C for 15 min. Deparaffinization, rehydration, antibody retrieval, titration, and visualization were fully automated by the Ventana Benchmark Ultra Slide Stainer (Ventana Medical Systems Inc., Tucson, AZ, USA). Duplex immunohistochemistry was performed with anti-CD8 (1:100, clone 8/144b, Dako, Agilent, Santa Clara, CA, USA) and pan-cytokeratin (1:2400, clone BS5, Monosan, Uden, the Netherlands). CD8 was visualized with DAB chromogen (Dako), and pan-cytokeratin was visualized with Ultraview Alkaline Phosphatase Red chromogen (Ventana Medical Systems). Hematoxylin was used as background stain.

2.5 | Digital Image Analysis

Slides were digitized with a Philips IntelliSite Ultra-Fast Scanner (Philips Digital Pathology Solutions) at 40× magnification with a resolution of 0.25 µm/pixel. The scans were further processed with QuPath image analysis software, version 0.2.0-m8 (Queen's University, https://qupath.github.io/) [16]. To calculate the tumor–stroma percentage, the tumor was divided into tumor–epithelium and tumor–stroma compartments, based on the pan-cytokeratin staining. In QuPath, the image settings were set at "brightfield (other)." The values were chosen so that the chromogens could be deconvoluted correctly into the hematoxylin (blue), DAB (brown), and red (red) vector stains (Table S1). The tumor area on the slide was annotated manually and divided into tiles of 0.5×0.5 mm. Each tile was subdivided into a tumor–epithelium (positive for pan-cytokeratin, red color) and tumor–stroma (negative for pan-cytokeratin) compartment with the "Cytokeratin annotation creation" tool in QuPath. The settings were chosen so that luminal areas and mucinous lakes were excluded (Table S2). The same thresholds were applied to all images. The area of the tumor–epithelium and tumor–stroma compartment per tile was calculated. The tumor–stroma percentage was calculated by dividing the area of the tumor–stroma by the sum of the tumor–epithelium and tumor–stroma areas. The mean tumor–stroma percentage of all tiles was calculated per tumor. An arbitrary 50% cutoff of tumor–stroma percentage was used, similar to what has been used by Mesker et al. in CRC and validated in other studies [17–19].

2.6 | Statistical Analysis

Differences in overall survival were assessed using the Kaplan–Meier method and compared using the log-rank test. Univariate survival analyses were performed to correlate subgroups to clinicopathological variables by Fisher's exact tests. One-way ANOVA was used in case of the continuous variable age. Cox regression analyses were used to correlate subgroups with survival. $p < 0.05$ was considered to be statistically significant. All analyses were conducted using the program language R (version 3.6.1).

3 | Results

In total, 74 patients with resected DA were included. Their median age was 65.5 years, and 47 were males (63.5%). Most patients ($n = 52$, 70.3%) did not receive adjuvant chemotherapy. The clinicopathological features of these patients are reported in Table 1. Three different classification systems were applied, as described below, of which the overlap is shown in Figure 1.

3.1 | Classification by Chromosomal CNAs

Frequencies of chromosomal CNAs are shown in Figure 2A. Most common gains were observed for chromosomal arms 7p (30%–34%), 8 (both p and q) (31%–57%), 20q (28%–34%), and 13q (24%–33%), and most common losses for chromosome 9p (15%–28%), 17p (22%–35%), and 18q (12%–32%). These CNAs are commonly observed in tumors of the GI tract [20]. A cutoff of 25% of the genome altered was used and resulted in 39 (52.7%) CNA^{low} and 35 (47.3%) CNA^{high} tumors. Frequency plots for CNA^{low} and CNA^{high} tumors are shown in Figure 2B,C, respectively. In 19 CNA^{low} tumors, a GII of less than 10% was found. The tumor with the lowest GII was 0.05%, which means that only 0.05% of the genome harbored a CNA.

There was no difference in age at diagnosis, sex, administration of adjuvant treatment, lymph node involvement, or TNM stage between patients with CNA^{low} and CNA^{high} tumors.

Patients with CNA^{high} DA had a significantly shorter survival with a 5-year OS of 31.0% compared to 45.5% in patients with CNA^{low} DA (HR 2.20, 95% 1.12–4.30, $p = 0.02$, Figure S1).

3.2 | Classification by MSI Status

MSI-PCR revealed 16 (21.6%) MSI and 58 (78.4%) MSS tumors. MSI-PCR failed for one tumor, but was evaluated as mismatch repair proficient by IHC and thereby considered as MSS. All 16 MSI tumors were classified as CNA^{low}. Of 58 MSS tumors, 23 (39.7%) were CNA^{low}.

There was no difference in age at diagnosis, sex, administration of adjuvant treatment, lymph node involvement, or TNM stage between patients with MSS and MSI tumors.

Patients with MSI DA showed a non-significant trend towards longer survival with a 5-year OS of 60.8% compared to 33.1% in patients with MSS DA (HR 0.45, 95% CI 0.17–1.16, $p = 0.097$, Figure S2).

Multivariate analysis in all patients (with CNA status, TNM stage, and the use of chemotherapy as variables) resulted in a significantly better overall survival for patients with CNA-low compared to those with CNA-high DA (HR 2.54; 95% CI 1.20–5.35; $p = 0.01468$).

In the group of patients with MSS DA (58 patients), survival was not significantly different in patients with CNA^{high} and CNA^{low} tumors (HR 1.82; 95% CI 0.81–4.08; $p = 0.15$).

Multivariate analysis in patients with MSS DA only (with CNA status, TNM stage, and the use of chemotherapy as variables) resulted in a better overall survival for patients with CNA-low compared to those with CNA-high DA (HR 2.97; 95% CI 1.13–7.76; $p = 0.0266$).

3.3 | Classification by Tumor–Stroma Percentage

The tumor and stroma areas showed considerable heterogeneity across tumors, with a mean stroma percentage per tumor of

TABLE 1 | Clinicopathological characteristics of 74 patients with resected primary duodenal adenocarcinoma.

	Total	CNA-low	CNA-high	*p*	MSS	MSI-high	*p*	Stroma-low	Stroma-high	*p*
	n = 74	*n* = 39	*n* = 35		*n* = 58	*n* = 16		*n* = 51	*n* = 23	
Age				0.61			0.41			0.80
Mean (range)	64.42 (34–86)	65.03 (34–86)	63.74 (44–82)		64.97 (44–86)	62.44 (34–82)		64.63 (34–86)	63.96 (45–82)	
Sex				0.23			1			0.04
Male	47 (63.5%)	22 (56.4%)	25 (71.4%)		37 (63.8%)	10 (62.5%)		28 (54.9%)	19 (82.6%)	
Female	27 (36.5%)	17 (43.6%)	10 (28.6%)		21 (36.2%)	6 (37.5%)		23 (45.1%)	4 (17.4%)	
Adjuvant treatment				0.35			0.67			0.90
Chemotherapy	19 (25.7%)	9 (23.1%)	10 (28.6%)		16 (27.6%)	3 (18.8%)		14 (27.5%)	5 (21.7%)	
No chemotherapy	52 (70.3%)	27 (69.2%)	25 (71.4%)		39 (67.2%)	13 (81.3%)		35 (68.6%)	17 (73.9%)	
Unknown	*3 (4.0%)*	*3 (7.7%)*	*0 (0.0%)*		*3 (5.2%)*	*0 (0.0%)*		*2 (3.9%)*	*1 (4.4%)*	
Lymph node involvement				0.82			0.77			1
N0	30 (40.5%)	15 (38.5%)	15 (42.9%)		23 (39.7%)	7 (43.8%)		21 (41.2%)	9 (39.1%)	
N+	43 (58.1%)	23 (59.0%)	20 (57.1%)		35 (60.3%)	8 (50.0%)		29 (56.9%)	14 (60.9%)	
Unknown	*1 (1.4%)*	*1 (2.6%)*	*0 (0.0%)*		*0 (0.0%)*	*1 (6.3%)*		*1 (2.0%)*	*0 (0.0%)*	
TNM				0.87			0.55			0.39
Stage I	10 (13.5%)	4 (10.3%)	6 (17.1%)		8 (13.8%)	2 (12.5%)		9 (17.6%)	1 (4.3%)	
Stage II	16 (21.6%)	9 (23.1%)	7 (20.0%)		11 (19.0%)	5 (31.3%)		10 (19.6%)	6 (26.1%)	
Stage III	34 (46.0%)	18 (46.2%)	16 (45.7%)		27 (46.6%)	7 (43.8%)		24 (47.1%)	10 (43.5%)	
Stage IV	12 (16.2%)	7 (17.9%)	5 (14.3%)		11 (19.0%)	1 (6.3%)		7 (13.7%)	5 (21.7%)	
Unknown	*2 (2.7%)*	*1 (2.6%)*	*1 (2.9%)*		*1 (1.7%)*	*1 (6.3%)*		*1 (2.0%)*	*1 (4.3%)*	

Abbreviations: CNA, copy number alterations; MSI-high, microsatellite instable; MSS, microsatellite stable.

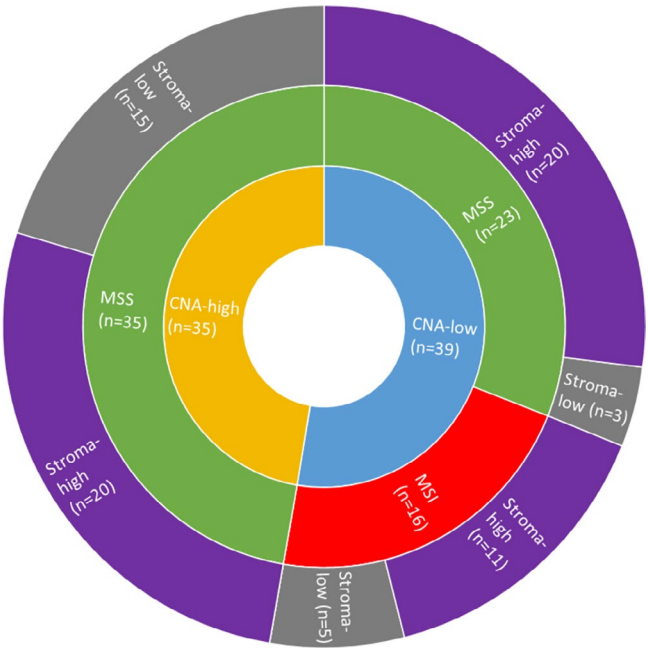

FIGURE 1 | Sunburst chart of the three classification methods. The distribution of DAs among classification methods is as follows: First, shallow whole genome sequencing resulted in 39 (52.7%) CNA^{low} and 35 (47.3%) CNA^{high} tumors. Overall, 16 (21.6%) DAs were MSI-high and 58 (78.4%) were MSS. The tumor–stroma content was low in 51 (68.9%) and high in 23 (31.1%) of DAs. Abbreviations: CNA, copy number aberrations; DA, duodenal adenocarcinoma; MSI-high, microsatellite instable; MSS, microsatellite stable.

43.2% and range between 8.9% and 91.1%. Using 50% as a cutoff for mean stroma percentage resulted in 51 (68.9%) stroma^{low} and 23 (31.3%) stroma^{high} tumors. Examples of tumors annotated as stroma^{high} or stroma^{low} are depicted in Figure S4.

The majority of patients with stroma^{high} tumors were male (83%), whereas the ratio of male (55%) to female (45%) patients was more equal in stroma^{low} tumors. There was no difference in age at diagnosis, administration of adjuvant treatment, lymph node involvement, or TNM stage between patients with stroma^{low} and stroma^{high} tumors.

Overall, there were no significant survival differences observed between patients with stroma^{high} DA with a 5-year OS of 34.7% compared to 41.3% in patients with stroma^{low} DA (HR 1.52, 95% CI 0.79–2.93, $p = 0.21$, Figure S3).

4 | Discussion

In this study, about half of the patients with DA (52.7%) were CNA^{low}, which was associated with longer overall survival compared to CNA^{high} DA. The rate of MSI tumors (21.6%) was higher compared to other gastrointestinal adenocarcinomas. As expected, MSI tumors also showed a trend towards a more favorable outcome in line with local colon cancer for example [5, 21]. This study provides new insights in a molecular classification of resected DA through enhancing our understanding of the molecular and histopathological characteristics of DA by analyzing CNAs, MSI status, and tumor–stroma content. This may contribute to tailor-made therapy and patient counseling in future patients with DA, especially since other studies have shown benefit of immunotherapy in not only MSI but also CNA^{low} tumors [22, 23].

This study shows that about half of patients with DA (52.7%) had CNA^{low} tumors, which is considerably higher than for GC and CRC, in which the majority (50%–88%) of tumors are CNA^{high} [3, 15]. It also shows that patients with CNA^{low} DA have a longer overall survival than those with CNA^{high} DA, independent of MSI status, which is in concordance with other gastrointestinal tumors [15]. The number of MSI tumors (21.6%) is higher in this study compared to other gastrointestinal adenocarcinomas but similar to DA, albeit its large range of reported frequencies (5%–35%) [5, 21, 24–27]. As expected, MSI tumors showed a trend towards a more favorable outcome, which is comparable to patients with resectable MSI GC or CRC [5, 28]. The limited number of patients included in the study could be a reason for not reaching statistical significance. The current study is the first to describe tumor–stroma percentage and its possible additional prognostic value for patients with DA. A possible hypothesis described before is that the stromal matrix has been shown to influence epithelial cell function in both malignant behavior and nonmalignant differentiation, with a change towards a higher stroma percentage regarded as ominous [17]. Furthermore, cancer-associated fibroblasts have been reported to have immune suppressive properties [29]. In previous studies, the tumor–stroma percentage has been shown to be an important prognostic parameter among patients suffering from different stages of CRC [17, 18, 30–34]. The current study cohort is the first cohort to describe tumor–stroma percentage and its possible additional prognostic value for patients with DA.

The relatively large number of MSI patients in the current cohort might have a positive effect on future treatment outcomes of DA, since neoadjuvant treatment with immunotherapy has shown promising results in patients with CRC [35]. In patients with locally advanced stage MSI CRC, neoadjuvant treatment with ipilimumab and nivolumab has shown a pathological response rate of 98% [35]. In addition, GC and CRC patients with CNA^{low} tumors have shown potential benefit from immunotherapy, regardless of MSI status [23]. Hence, immunotherapy might also be a future option for a substantial number of patients with DA [36]. The addition of immunotherapy to the treatment of DA should also be investigated, given the high percentage of patients with MSI tumors. The possibility of immunotherapy is of extra value, since the value of chemotherapy in MSI tumors has been topic of debate in both GC and CRC [37–40].

The results of the present study should be interpreted considering several limitations. First, DA is a rare disease, which limits its sample size. Second, Amsterdam UMC is a tertiary referral center for the treatment of DA, and all patients underwent a surgical resection. Selection bias may have affected the current study since Stage IV patients might be less frequently referred for surgery, and thus the study cohort could have consisted of patients in a relatively better clinical condition, which may have influenced survival data. Third, patients in this cohort received various systemic treatment regimens. Unfortunately,

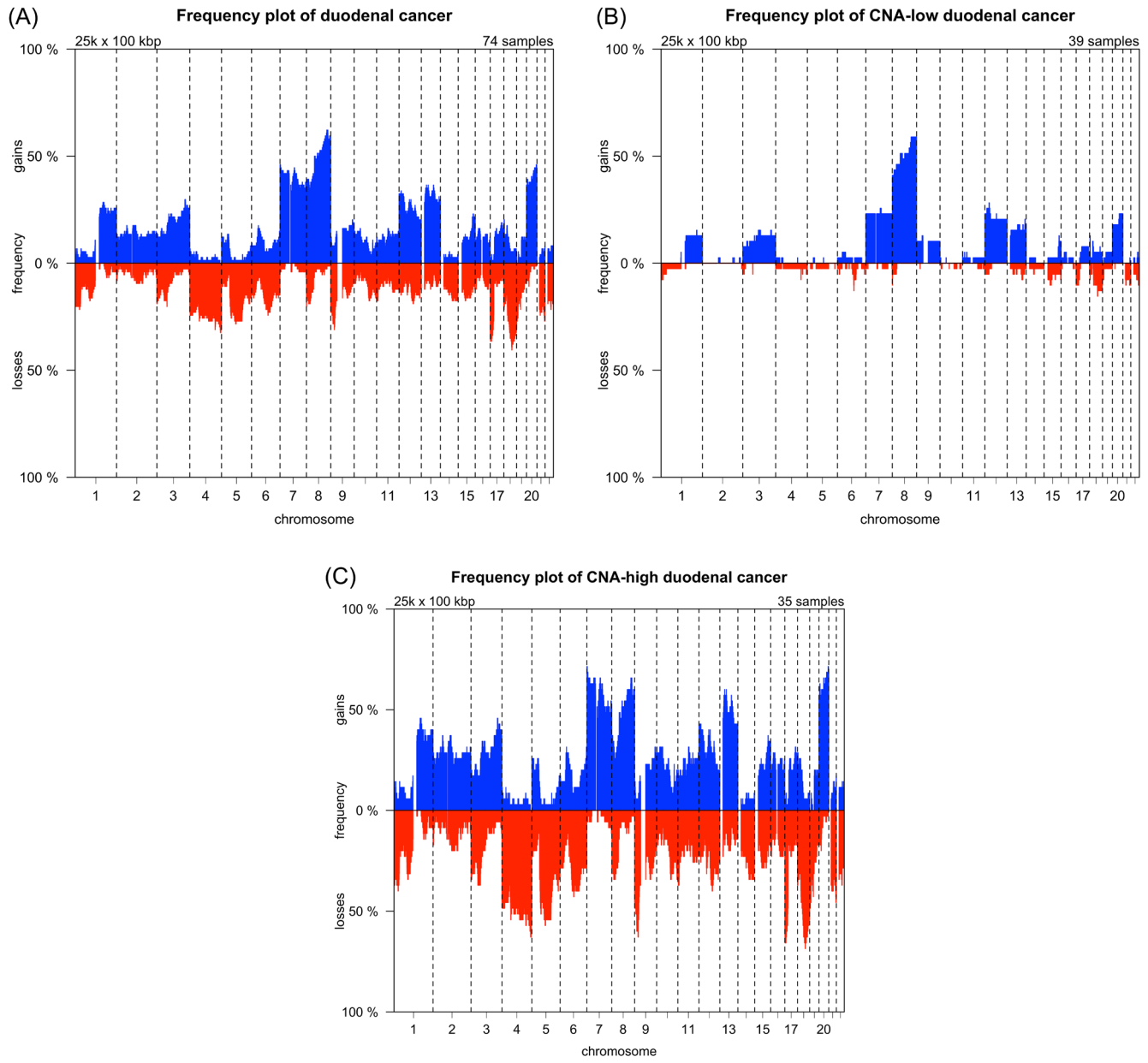

FIGURE 2 | Frequency plots of chromosomal CNAs for patients with resected DAs (A), in CNA^{low} (B), and CNA^{high} (C) duodenal adenocarcinomas. The frequency of gains is depicted in blue and losses in red.

no detailed information was available on the duration and type of chemotherapy or other systemic therapies, which could also have biased survival.

Further research should be performed to confirm the prognostic value of histopathological and molecular subtyping in DA. In addition, immunotherapy could be investigated in light of the relatively large amount of MSI and other CNA^{low} tumors. This may contribute to tailor-made therapy, including immunotherapy, for future patients with DA.

5 | Conclusion

Assessment of molecular subgroups of DA resulted in a relatively large number of MSI and other CNA^{low} tumors, when compared to other gastrointestinal adenocarcinomas. Patients with CNA^{low} DA have a significantly longer survival compared to patients with CNA^{high} DA, independent of MSI status. The large numbers of CNA^{low} tumors, and MSI in particular, make DA an interesting type of tumor to investigate the role of immunotherapy.

Author Contributions

J.B., H.B., T.S., J.E., N.G. and G.K. were involved in conception and design of the study, acquisition of data, analysis and interpretation of data, in drafting and revising critically for important intellectual content of all versions of the article, and gave final approval of this version of the manuscript to be published. J.B., H.B., T.S., J.E., T.B., M.B., L.V., B.Y., N.G. and G.K. were involved in conception and design of the study, interpretation of data, in revising critically for important intellectual content of all versions of the article, and gave final approval of this version of the manuscript to be published.

Ethics Statement

Ethical approval for this study series was obtained from Toetsingscommissie Biobanken Amsterdam UMC. Approval number 2014.038.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.