A clinically applicable molecular classification of oncocytic cell thyroid nodules

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Abstract

Whole-chromosome instability with near-whole genome haploidization (GH) and subsequent endoreduplication is considered a main genomic driver in the tumorigenesis of oncocyte cell thyroid neoplasms (OCN). These copy number alterations (CNA) occur less frequently in oncocyte thyroid adenoma (OA) than in oncocyte carcinoma (OCA), suggesting a continuous process. The current study described the CNA patterns in a cohort of 30 benign and malignant OCN, observed using a next generation sequencing (NGS) panel that assesses genome-wide loss of heterozygosity (LOH) and chromosomal imbalances using 1,500 single nucleotide polymorphisms (SNP) across all autosomes and the X chromosome in DNA derived from cytological and histological samples. Observed CNA patterns were verified using multiparameter DNA flow cytometry with or without whole-genome SNP array analysis and lesser-allele intensity-ratio (LAIR) analysis. On CNA-LOH analysis using the NGS panel, GH type CNA were observed 4 of 11 (36%) OA and 14 of 16 OCA (88%). Endoreduplication was suspected in 8 of 16 (50%) OCA, all with more extensive GH type CNA (p<0.001). Reciprocal chromosomal imbalance type CNA, characterized by (imbalanced) chromosomal copy number gains and associated with benign disease, were observed in 6 of 11 (55%) OA and one equivocal case of OCA. CNA patterns were different between the histopathological subgroups (p<0.001). By applying the structured interpretation and considerations provided by the current study, CNA-LOH analysis using an NGS panel that is feasible for daily practice may be of great added value to the widespread application of molecular diagnostics in the diagnosis and risk stratification of OCN.
Introduction

Thyroid oncocytic cells are follicular-derived oncocytic cells that are characterized by granular, eosinophilic cytoplasm due to an abundance of mitochondria. Their nuclei are enlarged and rounded, with prominent nucleoli (Wong, et al. 2020). Before the introduction of the 2023 WHO classification referred to as Hürthle cell neoplasms, oncocytic cell neoplasms (OCN) are defined as thyroid neoplasms composed of >75% oncocytic cells and include oncocytic thyroid adenoma (OCA) and carcinoma (OCA) (Baloch, et al. 2022; Lloyd, et al. 2017; WHO Classification of Tumours Editorial Board, 2022). OCN are distinct from other types of thyroid nodules or differentiated thyroid carcinoma in their biological and clinical behavior. OCA, in particular widely invasive OCA, typically show more aggressive behavior and less favorable prognosis than their non-oncocytic cell follicular counterparts, including higher rates of extra-thyroidal extension, radioiodine-refractory disease, distant metastases, and mortality (Goffredo, et al. 2013; Zhou, et al. 2020). Historically defined as a subtype of follicular thyroid carcinoma, the 2017 WHO classification has recognized OCA as a completely separate entity in follicular neoplasia (Lloyd, et al. 2017). Distinguishing benign from malignant OCN may be complex: cytological differentiation is not possible as (histopathological) assessment of capsular and vascular invasion is required, interobserver variability is observed, and metastases of OCN with an initial morphological diagnosis of OCA have been described (Boronat, et al. 2013; Cibas and Ali 2017; Grant, et al. 1988; Thodou, et al. 2021). In addition, oncocytic cell metaplasia/hyperplasia in the thyroid should be clearly distinguished from true OCN, as it is different in both origin and genetic alterations. It may occur in lymphocytic thyroiditis, oncocytic variant papillary thyroid carcinoma, medullary thyroid carcinoma, parathyroid lesions, and non-thyroid metastasis (Asa and Mete 2021; Thodou, et al. 2021).

Whole-chromosome instability is a main characteristic of the tumorigenesis in OCN. The process might be driven by a continuous redox imbalance due to the accumulation of malfunctioning mitochondria causing mitotic errors, leading to near-whole genome haploidization (GH, i.e., consistent with an A0 genotype) with subsequent endoreduplication (i.e., genome doubling, an AA genotype or
multiple thereof). These copy number alterations (CNA) seem to occur stepwise and in patterns in the progression from OA to OCA (Corver, et al. 2018). More extensive loss of heterozygosity (LOH) and endoreduplication are associated with progression of disease and worse prognosis (Corver, et al. 2014; Corver, et al. 2012; Doerfler, et al. 2021; Ganly, et al. 2018; Gopal, et al. 2018; Jalaly and Baloch 2020; Wada, et al. 2002). Corver et al. previously described the LOH of chromosomes 1, 2, 8, 9, 18 and 22 as early events and the additional loss of chromosomes 2, 3, 6, 11, 14-16, and 21 as later events indicating progression of disease, with the loss of chromosomes 1-4, 6 and 11 as the minimum signature of OCA (Corver, et al. 2018). Similar patterns were observed by Ganly et al., who described the LOH of chromosomes 2, 9, 11 and 18 as early events (Ganly, et al. 2018). The LOH of chromosome 7 is never observed, likely due to maternal- and paternal-imprinted genes important for survival (Boot, et al. 2016). Chromosomes 5, 12 and 20 are also frequently spared (Corver, et al. 2018; Ganly, et al. 2018). To a lesser extent, these characteristic CNA patterns are also described in part of the OA (Corver, et al. 2018; Doerfler, et al. 2021; Stankov, et al. 2004; Tallini, et al. 1999; Wada, et al. 2002). Other, reciprocal patterns of imbalanced chromosomal gains, consistent with genotype AAB, have also been observed in OA (Corver, et al. 2018; Wada, et al. 2002). In addition, driver point mutations (e.g., TERT promoter or TP53) have also been described in OCA (Corver, et al. 2018; Doerfler, et al. 2021; Ganly, et al. 2013; Ganly, et al. 2018; Jalaly and Baloch 2020; Kumari, et al. 2020; Santana, et al. 2020).

To advance the diagnostic workup and risk stratification of OCN, the development of a molecular test that is both accurate and feasible in everyday clinical practice is crucial. The methods previously used for CNA and LOH (CNA-LOH) analysis by Corver et al. and Ganly et al. are highly accurate and

The current study aimed to implement a clinically applicable test using a limited 1,500 SNP next generation sequencing (NGS) panel. We described the CNA patterns that we observed in benign and malignant OCN and assessed them in a clinical cohort of OCN, with certain focus on the methodological and bioinformatical aspects. We highlighted two cases to illustrate the added value of CNA-LOH analysis in clinical practice. Finally, we provided considerations for the structured interpretation of the CNA-LOH analysis results using this NGS panel.

**Materials and Methods**

**Study design and case selection**

For the current retrospective study, pseudonymized pathology records were reviewed to identify cases with a OCN of the thyroid in which molecular analysis including CNA-LOH analysis was performed
on the primary tumor during clinical practice at our tertiary care center between 1 May 2020 and 31 December 2021. Patients were only eligible for inclusion if the diagnosis of a oncocytic cell lesion was confirmed by histopathology, including nodular hyperplasia with oncocytic cell metaplasia (NH-H), OA, and OCA. Prior to inclusion in the current study, all histopathological diagnosis were made or reviewed by an experienced thyroid pathologist (HM) in accordance with the WHO classification (5th edition), including a morphological assessment and immunohistochemistry of H&E sections (WHO Classification of Tumours Editorial Board, 2022). Ethical study review was waived by the medical ethical review committee Leiden the Hague Delft (no. G21.167). No informed consent was required.

**Data collection**

From the pathology records, we recorded cytopathological and histopathological characteristics. Patient demographics and clinical, radiological, and surgical characteristics were collected from the patient medical records. Thyroid cytopathology was described using the Bethesda classification (Cibas and Ali 2017). The Thyroid Imaging Reporting and Data System (TIRADS) classification was infrequently reported and it was considered inappropriate to retrospectively reassess stored ultrasound captures, as ultrasound is a dynamic technique (Tessler, et al. 2017). Follow-up data was updated until 1 October 2022.

**Molecular analysis**

For molecular testing, total nucleic acid (DNA and RNA) was isolated either from formalin-fixed paraffin-embedded (FFPE) histopathology samples using FFPE tissue cores (0.6 mm diameter and variable length), from micro-dissected cytology cell blocks, or from tumor cells scraped off cytology slides (Cohen, et al. 2020; van der Tuin, et al. 2019a; van der Tuin, et al. 2019b). NGS was performed on the Ion Torrent GeneStudio™ S5 platform (GenomeScan BV, Leiden, The Netherlands) using custom NGS panels for CNA-LOH analysis, somatic DNA analysis, and gene fusion analysis. CNA-LOH analysis was performed using the custom AmpliSeq™ NGS genome-wide LOH (GWLOH) v2 panel, which assesses LOH and other chromosomal imbalances using 1,500 SNPs, evenly distributed across all autosomes and the X chromosome. This analysis has a transit time of only a few days. The
results of the GWLOH panel are visualized using SNP array plots (Figure 1) displaying the median amplicon read count (Figure 1A-D, top panel) visualizing the quality of the tested sample (>2.0 \[\log_{10}(100) = 2\] is considered good quality), the normalized median amplicon read count (middle panel) visualizing the relative copy number information, and the variant allele frequency (VAF) plot (bottom panel) visualizing any chromosomal imbalances including LOH. A VAF of 0.50 indicates heterozygosity; distinct segregation of the SNPs indicates chromosomal imbalance. Somatic mutation analysis was performed using the custom Ampliseq™ Cancer Hotspot v6 panel (Thermo Fisher Scientific, Waltham, MA, USA) or the custom Ampliseq™ NGS ENDO32 v1 panel (Thermo Fisher Scientific), which respectively assess 87 and 27 (thyroid) cancer-related genes, as previously described (details provided in Supplementary Data) (Cohen, et al. 2020; van der Tuin, et al. 2019a). Any alterations classified as (likely) pathogenic (i.e., class 4 or 5, respectively) were reported (Plon, et al. 2008). Gene fusion analysis was performed using the Archer® FusionPlex CTL v1 or v2 panel (ArcherDX Inc., Boulder, CO, USA), which respectively assess fusions in 16 and 19 genes, as previously described (Supplementary Data) (Cohen, et al. 2020; van der Tuin, et al. 2019a; van der Tuin, et al. 2019b).

CNA patterns

The results of the CNA-LOH analysis using the GWLOH panel were interpreted as described in the flowchart in Figure 2, identifying the different types of CNA patterns and establishing a molecular diagnosis. First, the CNA type is identified as GH type, or reciprocal chromosomal imbalance (RCI) type, or no CNA. GH type CNA are defined by LOH and chromosomal losses, as observed by chromosomal imbalances on the VAF plot (Figure 1B and 1C, bottom panel) and a lower normalized median amplicon read count of the affected chromosomes as compared to the unaffected chromosomes, indicating copy number losses (Figure 1B and 1C, middle panel). This CNA pattern requires further characterization by assessing the number of affected chromosomes, any heterogenicity of the alterations, and the possible presence of endoreduplication (Figure 2). We defined three categories for the number of affected chromosomes: 1-5, 6-10, and 11-23. Heterogenicity is defined as varying VAF amplitudes of the chromosomal imbalances among the affected chromosomes.
1B, bottom panel) and is associated with benign disease. Endoreduplication is also assessed using the VAF plot amplitudes. GH type CNA with (suspected) endoreduplication (Figure 1B) are characterized by an extreme amplitude of the SNP segregation of the LOH-affected chromosomes (Figure 1B, bottom panel). GH type CNA without endoreduplication (Figure 1C) are characterized by other, non-extreme VAF amplitudes of the chromosomal imbalances (Figure 1C, bottom panel). As the former is associated with OCA, primarily higher stage disease, and the latter is observed in both OA and OCA, it is important to aim to distinguish these two clinically relevant subtypes of the GH type CNA pattern (Corver, et al. 2018; Corver, et al. 2014; Corver, et al. 2012; Doerfler, et al. 2021; Ganly, et al. 2018; Gopal, et al. 2018; Jalaly and Baloch 2020; Stankov, et al. 2004; Tallini, et al. 1999; Wada, et al. 2002).

RCI type CNA (Figure 1D) are characterized by (imbalanced) chromosomal copy number gains, as observed by an SNP imbalance on the VAF plot (Figure 1D, bottom panel) and a higher normalized median amplicon read count of the affected chromosomes (Figure 1D, middle panel), indicating copy number gains. In contrast to GH type CNA, all chromosomes may be involved. No further characterization of RCI type CNA are required (Figure 2). The RCI type, consistent with genotype AAB, is foremost associated with benign oncocytic cell proliferations (Corver, et al. 2018; Wada, et al. 2002). No CNA are observed when the VAF plot indicates heterozygosity (Figure 1A, bottom panel) and the normalized median amplicon read count is similar across all chromosomes (Figure 1A, middle panel).

Next, the results of somatic mutation and fusion analysis are considered alongside the results of the CNA-LOH analysis (Figure 2). When additional driver mutations that are associated with OCA (e.g., TERT promoter, TP53) are observed, a malignant molecular diagnosis is considered more likely. Finally, the molecular diagnosis is categorized as (likely) benign, uncertain malignant, or malignant. We predefined a (likely) benign molecular diagnosis as limited GH type CNA, with 1-5 affected chromosomes, possible heterogeneity of the alterations, and no signs of endoreduplication, or any
RCI type alterations, or no molecular alterations on CNA-LOH, somatic mutation, and fusion analysis.

An uncertain malignant diagnosis was predefined as GH type alterations in 6-10 chromosomes or limited GH type alterations in 1-5 chromosomes in combination with an OCA-associated driver mutation. Heterogeneity of the alterations may be present among the affected chromosomes; no or inconclusive signs of endoreduplication are observed. A malignant molecular diagnosis is defined as extensive GH type CNA affecting 11-23 chromosomes, often with limited or without heterogenicity, or GH type alterations in 6-10 chromosomes in combination with an OCA-associated driver mutation, or GH type CNA with any number of affected chromosomes and suspected endoreduplication.

CNA pattern verification

To verify the GH type CNA patterns and appurtenant genotypes that are distinguished using the GWLOH v2 panel, CNA-LOH analysis using the GWLOH panel was performed on four representative historical cases (three OCA and one anaplastic thyroid carcinoma with oncocytic changes, not included in the study cohort) in which LAIR analysis was previously performed according to the methods as previously described by Corver et al. (Corver, et al. 2018). These included two cases with GH type CNA with suspected endoreduplication of the near-haploid genome (i.e., AA genotype) and two cases with GH type CNA without endoreduplication (i.e., A0 genotype). For each of these historical cases, the findings of the GWLOH panel were consistent with the findings of the LAIR analysis (Figure 3, Supplementary Data).

In addition, multiparameter DNA flow cytometry was performed according to the methods as described by Corver et al. (Corver, et al. 2005; Corver, et al. 2018), with minor modifications, on FFPE material of two cases from the study cohort: one OCA case (Table 2, case 29) with GH type CNA (Figure 4) and one OA case (Table 2, case 9) with RCI type CNA (Figure 5). In short, 2 mm tissue punches, taken from the same tissue block as used for the GWLOH panel, were transferred to a recipient paraffin block. Sixty-micron sections were taken, deparaffinized, heat-treated and dissociated using a mixture of collagen I and dispase with the aid of a gentleMACSTM (Miltenyi BioTec, Bergisch Gladbach, Germany). Next, cell suspensions were labeled for keratin (FITC), vimentin (APC) and DNA by propidium iodide (PI). Cells were treated with RNase to improve the resolution of the DNA
histograms. Cells were interrogated using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) (Corver, et al. 2005). For the historical cases (Figure 3, Supplementary figure 1), a LSRII flow cytometer (BD Biosciences, San Jose, CA) was used. All multiparameter DNA content flow cytometry data were analyzed using ModFit™ 6.0, remotely controlled by WinList™ 3D 10.0 (Verity Software House, Topsham, ME). The median relative fluorescence of the vimentin-positive, keratin-negative G₀G₁-fraction was used as internal DNA-diploid reference (Corver, et al. 2011; Corver, et al. 2005).

**Statistical analysis**

Where appropriate, parametric and nonparametric data were compared using the one-way ANOVA, and Mann-Whitney U or Kruskall-Wallis test, respectively. P values from the post-hoc analysis were adjusted by Bonferroni correction for multiple tests. Categorical data were compared using the 2-sided Pearson’s chi-squared or Fisher’s exact test, where appropriate. A p value of < 0.05 was considered statistically significant. All statistical analysis were performed using SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA).

**Results**

**Study cohort baseline**

Forty-six patients with CNA-LOH analysis using the GWLOH v2 panel on 48 nodules were screened for eligibility. Fourteen (29%) nodules, all with an ultrasound size smaller than 40 mm, were excluded because no surgery was performed, mainly due to the patients’ age and comorbidities. Four (9%) nodules with CNA-LOH analysis on (oncocytic cell) cytology were excluded because subsequent histopathology revealed a non-oncocytic cell lesion. Finally, 29 patients with 30 nodules were included (Table 1), including three (10%) with a histopathological diagnosis of NH-H, 11 (37%) OA, and 16 (53%) OCA including 13 (43%) minimally invasive (mi-OCA) and three (10%) widely invasive OCA (wi-OCA). There were no nondiagnostic CNA-LOH results.
CNA-LOH analysis

On CNA-LOH analysis using the GWLOH panel, CNA were observed in 25 of 30 (83%) lesions, including 10 of 11 (91%) OA, 12 of 13 (92%) mi-OCA, and 3 of 3 (100%) wi-OCA (Table 2, Figure 6). The CNA patterns were different between the histopathological subgroups (p<0.001). GH type CNA was found in 18 nodules, including 4 of 11 (36%) OA, 11 of 13 (85%) mi-OCA, and all wi-OCA. GH type CNA most frequently included chromosomes 2, 9, and 22, followed by 1, 3, 4, 6, 8, and 14. Chromosomes 7 and 12 were never involved. More chromosomes were affected in OCA (median 12 [IQR 7-16]) than OA (4 [3-9], p=0.04), but not in mi-OCA (12 [5-15]) as compared to wi-OCA (17 [8-17], p=0.06). Possible endoreduplication was observed in eight lesions, including one patient with American Joint Committee on Cancer stage I, four with stage III, and three with stage IVc disease (p=0.009) (Haugen, et al. 2016). The median number of affected chromosomes in lesions with suspected endoreduplication was higher (15 [IQR 12-17]) than in lesions without (4 [4-9], p<0.001). (Possible) heterogenicity was observed in 4 of 4 (100%) OA and 5 of 14 (36%) OCA with GH type CNA (p=0.08). RCI type CNA were observed in seven nodules, including 6 of 11 (55%) OA and one equivocal case of mi-OCA (Table 2, case 16, further discussed below). CNA were detected as the only genetic alteration in 12 of 16 (75%) OCAs. Three mi-OCA and one wi-OCA showed an additional molecular driver, including one EIF1AX, two TERT promoter, and one TP53 mutation. Morphological and molecular diagnoses were discordant case 14, 15, 16 (illustrated below), 17, 18, and 23 (illustrated below) (Table 2, Figure 6).

The histopathological nodule size was similar in benign and malignant nodules (p=0.19), but significantly different between CNA patterns (p=0.02). Post-hoc analysis showed that this difference was based on a larger size in nodules with GH type CNA with suspected endoreduplication than in GH type nodules without endoreduplication (p=0.01). Nodule size was similar between other CNA patterns.

Clinical cases
Case 23 (Table 2) was a 57-year-old male patient, who was referred to our tertiary care center with lymphatic, pulmonary, and bilateral renal metastases of an OCA. Twelve years earlier, he had undergone a diagnostic hemithyroidectomy for a right-sided thyroid nodule. At the time, it was morphologically diagnosed as an OA and no further treatment was considered necessary. Recent review of the histopathology slides supported the original morphological diagnosis, showing a 27-mm OCN with large nucleoli, (pseudo-)papillary architecture without papillary nuclear features, and a thin capsule, without signs of capsular or vascular invasion (Figure 7a and 7b). A biopsy of the pulmonary metastasis showed similar histopathological characteristics (Figure 7c and 7d), indicating that, in retrospect, the known OCN should likely be considered an OCA (T2N1bM1). CNA-LOH analysis using the GWLOH panel was performed on the biopsy of the pulmonary metastasis and on preserved FFPE material of the primary tumor. The primary tumor showed extensive GH type CNA with high suspicion of endoreduplication, involving chromosomes 1-4, 6, 8, 9, 11, 14, 15, 20-22 (Figure 1B), also denoting the malignant nature of the lesion. No point mutations or gene fusions were found. A similar CNA pattern was observed in the pulmonary metastasis, confirming its origin. No contralateral thyroid tumor was present. The patient recently underwent completion thyroidectomy and adjuvant radioiodine therapy.

Case 16 (Table 2) was a 63-year-old female patient, who presented with a palpable, left-sided thyroid nodule from which Bethesda IV cytology was obtained. She underwent a diagnostic hemithyroidectomy, revealing a 20-mm OCN with a thick capsule and without signs of vascular invasion. On histopathological assessment, the tumor capsule showed a focal interruption of equivocal invasive malignant or iatrogenic origin. Immunohistochemistry showed no loss of PTEN protein expression and wild-type p53 expression. Based on the possible capsular invasion, the neoplasm was initially considered a mi-OCA (T1bN0Mx). CNA-LOH analysis showed RCI type CNA of chromosomes 1, 5, 6, 10, 12, 16, 17, 19, 20, and X (Figure 1D). No somatic mutations or gene fusions were found using the Ampliseq™ Cancer Hotspot v6 and the Archer® FusionPlex CTL v2 panels. As such, this OCN was molecularly diagnosed as (likely) benign. The patient and her endocrinologist agreed to a follow-up regimen, refraining from completion thyroidectomy. Thus, the patient
underwent a dedicated neck ultrasound every six months, to date revealing no abnormalities in 38 months of follow-up. The patient currently remains in follow-up.

**Discussion**

In the current study, we described the CNA patterns that were observed on CNA-LOH analysis in benign and malignant OCN using a limited, 1,500 SNP GWLOH NGS panel. With this method that is feasible for daily clinical practice, we are able to distinguish four CNA patterns and establish a molecular diagnosis with an increasing risk of malignancy. The assessment of the CNA patterns in a patient cohort confirmed that GH type CNA is found primarily in OCA but to a lesser extent also in OA. GH type alterations with possible endoreduplication were reserved for OCA, mi-OCA as well as wi-OCA, and mainly those with more extensive chromosomal losses. These results are in accordance with previous studies that reported on the early and late events in near-whole genome haploidization (Corver, et al. 2018; Corver, et al. 2014; Corver, et al. 2012; Doerfler, et al. 2021; Ganly, et al. 2018; Gopal, et al. 2018; Jalaly and Baloch 2020). Only the loss of chromosome 18 was infrequently observed in the current study, in contrast to observations by Corver et al. and Ganly et al. (Corver, et al. 2018; Ganly, et al. 2018; Gopal, et al. 2018). RCI type CNA, which have less frequently been described in literature, were foremost associated with benign disease in the current study (Corver, et al. 2018; Wada, et al. 2002). Point mutations were rarely observed and gene fusions were not found in our cohort, endorsing that CNA are likely the main molecular drivers in OCN.
CNA-LOH analysis may be of great added value in the (preoperative) diagnosis and risk stratification of OCN in clinical practice, aiding multidisciplinary patient management decisions. Case 23 illustrated that CNA-LOH analysis can be pivotal in identifying aggressive biological potential in OCN, especially if morphological histopathological features of malignancy are lacking. Although infrequently reported, metastases of OCN that were initially morphologically diagnosed as OA are notorious (Boronat, et al. 2013; Grant, et al. 1988). In hindsight, based on the molecular profile of the original tumor, treating the original lesion of case 23 as an OCA would have been justified. Unfortunately, these molecular techniques were not available yet at the time of the original diagnosis. Case 16 illustrated that CNA-LOH analysis may also contribute to the risk reduction and avoid possible overtreatment of OCN with an equivocal histopathological diagnosis of OCA. Based on the molecularly benign CNA pattern that was observed in this case, indolent biological behavior or even a benign nature of the neoplasm may be expected (Corver, et al. 2018).

Following the results of the current and previous studies, initial total thyroidectomy could be considered instead of diagnostic hemithyroidectomy for oncocytic cell lesions with (extensive) GH type alterations and (suspected) endoreduplication (i.e., a malignant molecular diagnosis) (Corver, et al. 2014; Corver, et al. 2012; Doerfler, et al. 2021; Ganly, et al. 2018; Gopal, et al. 2018; Jalaly and Baloch 2020). In less extensive GH type alterations without (suspected) endoreduplication (i.e., an uncertain malignant molecular diagnosis), diagnostic hemithyroidectomy is recommended to obtain a definitive diagnosis. When RCI type CNA are observed, hemithyroidectomy should also be considered. Although associated with biologically benign disease in the current study, evidence regarding RCI type CNA is still limited and future studies are needed to confirm our observations (Corver, et al. 2018; Wada, et al. 2002).

Finally, the absence of CNA alone does not exclude malignancy and does not justify withholding diagnostic hemithyroidectomy for a Bethesda III/IV oncocytic cell nodule. Instead, the results of the CNA-LOH analysis are best interpreted in combination with somatic mutation and fusion analysis results (Figure 2). Various point mutations have additionally been described in OCA, infrequently in
malignancies lacking the typical GH type CNA patterns. These include DAXX, NF1, ARHGAP35, MADCAM1, ATXN1, UBXN11, TSC1/2 and CDKN1A mutations, mutations characteristic of FTC including RAS, PIK3CA, and PTEN mutations, and those that are characteristic of poorly differentiated and anaplastic thyroid carcinoma including TERT promoter, PIK3CA, PTEN, EIF1AX, ATM, and TP53 (Corver, et al. 2018; Doerfler, et al. 2021; Ganly, et al. 2013; Ganly, et al. 2018; Jalaly and Baloch 2020; Kumari, et al. 2020; Santana, et al. 2020). As in other types of thyroid carcinoma, TERT promoter mutations in OCA are associated with more aggressive tumor behavior, distant metastasis, and tumor dedifferentiation including radioiodine refractory disease (Ganly, et al. 2018). The somatic mutation and gene fusion NGS panels that were used in the current study included the most important but not all of the OCA-appurtenant molecular alterations that were previously described in literature. Yet, when no CNA and no somatic mutations or gene fusions are observed, a wait-and-see strategy with active surveillance of the nodule appears oncologically safe and should be considered.

During CNA-LOH analysis using the GWLOH panel, a number of additional considerations are crucial (Box 1). First, to establish the diagnostic value of the CNA-LOH analysis, ascertaining the presence of true oncocytes as opposed to oncocytic cell metaplasia is key (WHO Classification of Tumours Editorial Board, 2022). A careful morphological assessment (including immunohistochemistry) by a dedicated thyroid pathologist may accurately distinguish most true oncocytic cell lesions from other neoplastic or non-neoplastic disorders that present with oncocytic changes, such as oncocytic papillary thyroid carcinoma, oncocytic medullary thyroid carcinoma, or parathyroid proliferations (Asa and Mete 2021). Such neoplasms also show different genetic alterations (Doerfler, et al. 2021). The observation of an atypical CNA pattern or a somatic mutation or gene fusion that is uncommon in OCN, warrants the critical re-evaluation of the cell type and (non-)oncocytic cell origin of the tumor, including consideration of alternative diagnoses (Lloyd, et al. 2017).

Next, the tumor cell percentage of the tested tissue sample should always be taken into consideration. Whereas the tumor cell percentage of OCN is mostly 70-80% or higher for histopathological samples,
often ensuring clear amplitudes in the SNP array plots, the tumor cell percentage of cytology samples can be limited to 30-50%, resulting in smaller VAF amplitudes and/or more scattered SNP plots. In the latter cases, endoreduplication may present with less extreme amplitudes and may even go unnoticed in the assessment. As such, the GWLOH panel can indicate but not always exclude endoreduplication, and – dependent on the quality of the tissue sample – sometimes no decisive answer regarding the presence of endoreduplication may be obtained. Other techniques such as flow cytometry and LAIR analysis are more reliable for this purpose, but these are not fit for daily clinical application (Corver, et al. 2008; Corver, et al. 2012). Finally, although recognizing endoreduplication may seem critical due to its association with an unfavorable prognosis and metastatic disease, it is important to realize that metastasis have occasionally also been described for tumors without genome doubling (Corver, et al. 2018; Corver, et al. 2014; Corver, et al. 2012; Doerfler, et al. 2021; Ganly, et al. 2018; Gopal, et al. 2018; Jalaly and Baloch 2020).

Finally, CNA-LOH analysis results should be carefully interpreted in the context of other clinicopathological characteristics, including nodule size, for example. Larger nodule size (>4 cm, in particular) has previously been associated with a higher risk of malignancy in OCN and worse prognosis in OCA (Doerfler, et al. 2021; Lopez-Penabad, et al. 2003; Santana, et al. 2019). Although the current study found no statistically significant difference in nodule size between benign and malignant lesions, nodules with GH type CNA and possible endoreduplication were significantly larger than GH type nodules without endoreduplication.

CNA-LOH analysis may resolve important bottlenecks in the preoperative differentiation of OCN. Besides their unique molecular alterations, OCN oftentimes also show atypical results and lower diagnostic accuracy on other preoperatively applied diagnostics, including ultrasound and positron emission tomography/computed tomography (PET/CT) using 2-[18F]fluoro-2-deoxy-D-glucose (FDG) (de Koster, et al. 2018; de Koster, et al. 2022; Slowinska-Klencka, et al. 2020). FDG-PET/CT visualizes (increased) metabolic activity in tissues and is successfully applied for the diagnosis,
staging and monitoring of many types of cancers (Boellaard, et al. 2015). A visually negative FDG-PET/CT accurately differentiates between benign and malignant cytologically indeterminate thyroid nodules. This, however, does not apply to nodules with oncocytic cell cytology, which are almost exclusively strongly FDG-positive, likely related to their abundance of mitochondria (de Koster, et al. 2022).

Due to the increased use of imaging techniques for indications unrelated to the thyroid, thyroid nodules are detected in up to 65% of the general population (Bernet and Chindris 2021; Durante, et al. 2018). This includes FDG-positive thyroid incidentalomas, which are found in approximately 2% of FDG-PET/CT scans with an approximate 31% malignancy rate (de Leijer, et al. 2021). The exact number of oncocytic cell lesions among these incidentalomas is unknown, but may be substantial due to the pronounced FDG-positivity in these nodules (de Koster, et al. 2022; Pathak, et al. 2016). In our screened patient population, 11 of 48 (23%) presented with a PET/CT thyroid incidentaloma. Six of 11 (55%) patients did not undergo surgery, most frequently due to (oncological) comorbidities that were the indication for the FDG-PET/CT. In patients with considerable comorbidities, molecular testing including CNA-LOH analysis may aid the considerations and risks of withholding surgery.

The main limitation of our study is its retrospective design, potentially causing bias. Selection bias may have resulted in a nonrepresentative patient cohort if CNA-LOH analysis and/or thyroid surgery were only selectively performed. Moreover, some of the included patients were consultations from community hospitals, specifically referred to our hospital for CNA-LOH analysis. In addition, the relatively small sample size of our cohort limited further statistical analysis of the observed CNA patterns in relation to clinical and histopathological characteristics. The current study was not designed to assess diagnostic performance of the GWLOH panel. Larger, prospective validation studies are desired to explicate the CNA-appurtenant risk stratification of OCN and assess (preoperative) diagnostic accuracy parameters of CNA-LOH analysis, including differences in test performance between cytological and histopathological tissue specimens. Such cohorts could also include a wider range of neoplastic or non-neoplastic thyroid and parathyroid disorders that present with oncocytic
changes, to confirm the absence of OCN-characteristic CNA in these diagnoses. Validation studies from our study group are currently in progress, including molecular diagnostics of the prospective EffEcts trial cohort (ClinicalTrials.gov: NCT02208544)(de Koster, et al. 2022) and a separate prospective trial on preoperative risk stratification of cytologically indeterminate thyroid nodules using molecular diagnostics. Both include non-oncocytic and oncocytic nodules.

In addition, it currently remains unknown whether more accurate differentiation and risk stratification of OCN using molecular diagnostics, including consequent treatment decisions, finally improves the prognosis of OCA. This requires data that is not yet available, i.e., from large cohort studies with molecular diagnostics and follow-up extending over several decades. Finally, cost-effectiveness should be taken into consideration and formal cost-utility studies should be performed in the future.

The joint costs of our molecular panels are approximately €1,350 ($1,479; $1=€0.91 on 02-05-2023) per patient with an OCN.

In conclusion, the results of this study demonstrate that CNA-LOH analysis using a limited, 1,500 SNP NGS panel is a feasible method for CNA-LOH analysis in OCN in everyday clinical practice.

The results of this study, including full description of the CNA patterns that may be distinguished and considerations for their structured interpretation to establish a molecular diagnosis, may aid the widespread application of CNA-LOH analysis for the preoperative as well as postoperative diagnosis and risk stratification of oncocytic cell lesions.
Declaration of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Author contribution statement

HM and TW conceptualized the study. HM was the project leader. EK prepared the dataset for analysis, drafted the manuscript, and prepared the tables and figures. EK, WC, and HM verified the data and performed the statistical analysis. All authors contributed to data acquisition and the interpretation of the data, and critically reviewed this manuscript. All authors had full access to all the data in the study and approved the manuscript before submission. HM had final responsibility for the decision to submit for publication.
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Figure 1.
The main CNA patterns, observed by CNA-LOH analysis using the custom AmpliSeq™ NGS GWLOH v2 panel (GenomeScan BV, Leiden, The Netherlands), which assesses approximately 1,500 SNPs across all autosomes and the X chromosome. These SNP array plots show the median amplicon read count (A-D, top panel) visualizing the quality of the tested sample, the normalized median amplicon read count (A-D, middle panel) visualizing the relative copy number information, and the VAF (A-D, bottom panel) visualizing any chromosomal imbalances. Results are displayed per chromosome (see horizontal numbering in the top row) and separately for p (blue) and q arm (yellow).

**Figure 1A** displays a normal pattern with no CNA, as observed in nodular hyperplasia with oncocytic cell metaplasia (Table 2, case 3). **Figure 1B** displays the results of the CNA-LOH analysis performed on the histology (tumor cell percentage ≥70%) of an OCA in a male patient (Table 2, case 23), with GH type CNA with suspected endoreduplication, characterized by clear loss of heterozygosity (a, bottom panel) and chromosomal losses (B, middle panel) of chromosomes 1-4, 6, 8-9, 11, 14, 15 and 20-22. There was some heterogenicity, indicated by the smaller VAF amplitude of chromosomes 15 and 20 as compared to the other affected chromosomes (B, bottom panel). The extreme VAF amplitude (B, bottom panel) of most affected chromosomes is highly suspect of endoreduplication. This neoplasm was diagnosed as molecularly malignant. This case is further illustrated in the Results section. **Figure 1C** displays the results of the GWLOH panel performed on the histology (tumor cell percentage ≥70%) of an OA in a male patient (Table 2, case 12), with GH type CNA without endoreduplication, characterized by chromosomal imbalances with less pronounced amplitudes (C, bottom panel) and chromosomal losses (C, middle panel) of affected chromosomes 2, 9, 13, and 22. Limited heterogenicity is possibly observed. This OA was considered molecularly benign. **Figure 1D** displays an oncocytic cell neoplasm in a female patient (Table 2, case 16). On histopathological assessment, this lesion had a thick capsule and no signs of vascular invasion. Based on a focal interruption of the capsule of equivocal invasive malignant or iatrogenic origin, the neoplasm was considered a minimally invasive OCA. On CNA-LOH analysis, the lesion showed RCI type CNA, characterized by chromosomal imbalances (D, bottom panel) and relative chromosomal gains (D,
middle panel) affecting chromosomes 1, 5, 6, 10, 12, 16, 17, 19, 20, and X. As such, molecularly, this lesion is considered benign. This case is further illustrated in the Results section.

CNA, copy number alterations. CNA-LOH, copy number alterations and loss of heterozygosity. GH type, genome-haploidization type. GWLOH, genome-wide loss of heterozygosity. OA, oncocytic thyroid adenoma. OCA, oncocytic cell thyroid carcinoma. RCI type, reciprocal chromosomal imbalance type. SNP, single nucleotide polymorphism. VAF, variant allele frequency.
Figure 2.

Flowchart for the systematic interpretation of the SNP array plots visualizing the results of CNA-LOH analysis using the GWLOH v2 panel, in order to establish a molecular diagnosis. First, the CNA type is identified as GH type, RCI type, or no CNA. GH type CNA, defined by loss of heterozygosity and chromosomal losses, are further characterized by assessing the number of affected chromosomes, the presence of heterogenicity of the alterations among the affected chromosomes, and the possible presence of endoreduplication. RCI type CNA are defined by (imbalanced) chromosomal copy number gains. No further characterization of these alterations is needed. Next, the results of somatic mutation and fusion analysis should be considered alongside the results of the CNA-LOH analysis. Finally, the molecular diagnosis is determined as (likely) benign, uncertain malignant, or malignant. *: in case a somatic mutation or gene fusion is identified that is uncommon in oncocytic cell neoplasms, whether or not in combination with atypical CNA patterns, reevaluate the presence of true oncocytic cells in the sample (also see Box 1) and consider alternative diagnoses that may present with oncocytic cell metaplasia. †: including but not limited to TERT promoter or TP53 mutations. CNA, copy number alterations. CNA-LOH, copy number alterations and loss of heterozygosity. GH type, genome-haploidization type. GWLOH, genome-wide loss of heterozygosity. RCI type, reciprocal chromosomal imbalance type.
Figure 3.

Verification of the CNA patterns observed during CNA-LOH analysis using the GWLOH v2 NGS panel (F and L) in historical cases on which LAIR analysis (A-E and G-K) was previously performed according to the methods as described by Corver et al. (Corver, et al. 2018). One OCA (A-F) and one ATC-H (G-L) are presented here. A. The SNP array analysis (iCOG and HumanCytoSNP-12, Illumina, Inc., San Diego, CA, USA) of the OCA in a male patient showed homozygosity and chromosomal losses of chromosomes 1-4, 6, 8-11, 12q, and 13-22. B and H. Keratin (FITC) vs. vimentin (APC) fluorescence. Different populations R1 (vimentin-positive, keratin negative) and R2 (vimentin-positive and keratin-positive) can be identified. C and I. Negative control stained with the secondary reagents and PI only. Note the difference in fluorescence intensities as compared to keratin and vimentin-stained samples (B and H). D and J. Overlay of population R1 and R2. Note that the major G₀G₁-peak of the histogram is painted red and left of the green G₀G₁-peak (the DNA-diploid internal reference, minor in D and major in J), indicative for loss of DNA. E and K. The MFI of the G₀G₁-of R1 was used to accurately calculate the DNA index of the major G₀G₁-peak of R2. E. The DNA histogram of R2, the keratin-positive, vimentin-positive population showed debris for which the ModFit LT algorithm corrects. The DNA index was 0.7. A-E. Together, these findings indicated the loss of DNA and near-haploidy, corresponding to an A0 genotype. G. The iCOG SNP array of the ATC-H in a female patient showed homozygosity and chromosomal losses of chromosomes 1-6, 8-11, and 13-21. K. The DNA histogram of R2, the keratin-positive, vimentin-positive population showed to be bi-modal due to two cycling populations, with DNA indices of 0.5 (i.e., near-haploid tumor fraction) and 1.1 (i.e., fraction with endoreduplication), respectively. G-K. Together, these findings showed loss of DNA, near-haploidy and endoreduplication of the entire near-haploid population. F and L. On the GWLOH panel, both cases showed chromosomal losses of the affected chromosomes relative to the unaffected chromosomes (middle panels, normalized median amplicon read count). The VAF of the OCA (F, bottom panel) showed a less pronounced amplitude of the SNP segregation than the ATC-H (L, bottom panel), corresponding to GH type CNA without (F) and with (L) endoreduplication, respectively. As such, the CNA patterns observed using the GWLOH panel were
consistent with the results of the LAIR analysis. The two other historical cases are presented in Supplementary Figure 1.

488 or 633, laser wavelength used for excitation. 530/30, band pass filter used to collect FITC fluorescence (green). 670/14, band pass filter used to collect APC fluorescence (infra-red). >610/20, long pass filter used to collect PI fluorescence (deep-red). APC, allophycocyanin. ATC-H, anaplastic thyroid carcinoma with oncocyic changes. CNA, copy number alterations. CNA-LOH, copy number alterations and loss of heterozygosity. FITC, fluorescein isothiocyanate. GH type, genome haploidization type. GWLOH, genome-wide loss of heterozygosity. OCA, oncytic thyroid carcinoma. LAIR, lesser-allele intensity-ratio. MFI, median fluorescence intensity. RCI type, reciprocal chromosomal imbalance type. SNP, single nucleotide polymorphism. VAF, variant allele frequency.
**Figure 4.**

An 85 mm widely invasive oncocyctic thyroid carcinoma with GH type CNA (Table 2, case 29). **A and B.** Hematoxylin and eosin-stained sections (A, 0.5x, 2mm, and B, 40x, 50 μm) from a formalin-fixed, paraffin-embedded specimen of the thyroid tumor. Punches from the same tissue block were used for CNA-LOH analysis using the GWLOH panel and for multiparameter DNA content flow cytometry. **C.** Results of the GWLOH panel (tumor cell percentage at least 80%) showed GH type CNA, with losses of chromosomes 1, 2, 3, 4, 6, 8, 9, 10, 11, 14, 15, 16, 18, 21, and 22, some heterogeneity, and possible endoreduplication. **D-I.** Results of the multiparameter DNA content flow cytometry. **D.** Keratin (FITC) vs. vimentin (APC) fluorescence. Different populations R1 (vimentin-positive, keratin negative) and R2 (vimentin-positive and keratin-positive) can be observed. **E.** Negative control stained with the secondary reagents and PI only. **F.** DNA histogram of R1 (green), the vimentin-positive, keratin negative DNA-diploid (internal reference) stromal population. **G.** DNA histogram of R2 (red), the vimentin-positive, keratin-positive population epithelial cell fraction. **H.** Overlay of population R1 and R2. Note that the major G₀G₁-peak of the histogram is painted red and to the left of the green G₀G₁-peak (the DNA-diploid internal reference), clearly indicating loss of DNA and thus likely representing a near-haploid carcinoma population. **I.** The MFI of the G₀G₁-population of R1 calculated by WinList 3D was linked to ModFit LT allowing accurate calculation of the DNA index of the major G₀G₁-peak of R2. The DNA index was 0.7, confirming DNA near-haploidy and the GH type CNA pattern that was observed using the GWLOH panel. Although endoreduplication was deemed possible on the GWLOH panel, flow cytometry results indicated that no endoreduplication was present (i.e., A0 genotype). On somatic mutation and fusion analysis, no driver mutations were identified. This tumor was diagnosed as molecularly malignant.

488 or 633, laser wavelength used for excitation. 530/30, band pass filter used to collect FITC fluorescence (green). 660/20, band pass filter used to collect APC fluorescence (infra-red). >670, long pass filter used to collect PI fluorescence (deep-red). APC, allophycocyanin. CNA, copy number alterations. CNA-LOH, copy number alterations and loss of heterozygosity. FITC, fluorescein isothiocyanate. GH type, genome haploidization type. GWLOH, genome-wide loss of heterozygosity. MFI, median fluorescence intensity.
Figure 5.
A 58 mm oncocytic thyroid adenoma with RCI type CNA (Table 2, case 9). A and B. Hematoxylin and eosin-stained sections (A, 5x, and B, 40×) from a formalin-fixed, paraffin-embedded specimen of the tumor. Punches from the same tissue block were used for CNA-LOH analysis using the GWLOH panel and for multiparameter DNA content flow cytometry. C. The results of the GWLOH panel (tumor cell percentage at least 50%) showed chromosomal imbalances of chromosomes 5, 6, 7, 11, 12, 13, 17, 20 and X based on chromosomal copy number gains, corresponding to RCI type CNA. D. Keratin (FITC) vs. vimentin (APC) fluorescence, with R1 (vimentin-positive, keratin negative) and R2 (vimentin-positive and keratin-positive) populations. E. Negative control stained with the secondary reagents and PI only. F. DNA histogram of R1 (green), the vimentin-positive, keratin negative DNA-diploid (internal reference) stromal population. G. DNA histogram of R2 (red), the vimentin-positive, keratin-positive population epithelial cell fraction. H. Overlay of population R1 and R2. Note that the major G0G1-peak of the histogram is painted red and to the right of the green G0G1-peak (the DNA-diploid internal reference), indicative for gain of DNA. I. The MFI of the R1G0G1-population was used to accurately calculate the DNA index of the major G0G1-peak of R2. The DNA index was 1.3, indicating gain of DNA and aneuploidy. As such, the results of the multiparameter DNA content flow cytometry are in accordance with the results of the GWLOH panel. On somatic mutation and fusion analysis, no driver mutations were identified. This tumor was considered molecularly benign.

488 or 633, laser wavelength used for excitation. 530/30, band pass filter used to collect FITC fluorescence (green). 660/20, band pass filter used to collect APC fluorescence (infra-red). >670, long pass filter used to collect PI fluorescence (deep-red). APC, allophycocyanin. CNA, copy number alterations. CNA-LOH, copy number alterations and loss of heterozygosity. FITC, fluorescein isothiocyanate. GH type, genome haploidization type. GWLOH, genome-wide loss of heterozygosity. MFI, median fluorescence intensity. RCI type, reciprocal chromosomal imbalance type.
Figure 6.

A. Molecular alterations observed in 30 oncocytic cell nodules based on CNA-LOH analysis using the GWLOH v2 panel, and somatic point mutation and gene fusion analysis. Nodules 12 and 13 are a left- and right-sided nodule in the same patient. Although CNA patterns appear similar in both nodules, further analysis of the SNP profiles demonstrated that the GH type alterations appeared in different alleles on chromosomes 13 and 22 of the two nodules. The lesions were therefore considered of different clonal origin and both included in the current study. B. Clustered bar chart showing the statistically significantly different rate of the CNA patterns among the different histopathological diagnoses (p<0.001). CNA, copy number alterations. GH type, genome haploidization type. GWLOH, genome-wide loss of heterozygosity. OA, oncocytic thyroid adenoma. mi-OCA, minimally invasive oncocytic thyroid carcinoma. NH-H, nodular hyperplasia with oncocytic cell metaplasia. RCI type, reciprocal chromosomal imbalance type. Wi-OCA, widely invasive oncocytic thyroid carcinoma.
Figure 7.

Case 23 (Table 2). A and B. Hematoxylin and eosin-stained sections of the primary thyroid tumor, a 27-mm oncocytic cell neoplasm (A, 0.25x and B, 60x) showing large nucleoli, (pseudo-)papillary architecture without papillary nuclear features, and a thin capsule with no signs of capsular or vascular invasion. It was initially morphologically diagnosed as a oncocytic thyroid adenoma and treated as such. Twelve years later, the patient presented with a pulmonary metastasis of this neoplasm (Hematoxylin and eosin-stained sections C, 10x and D, 60x). Results of the CNA-LOH analysis of this patient are presented in Figure 1B.
Figure 1.

170x225mm (600 x 600 DPI)
Figure 1 (continued).

170x82mm (600 x 600 DPI)
Figure 2.

243x131mm (300 x 300 DPI)
Figure 3.

170x225mm (600 x 600 DPI)
Figure 4.

170x205mm (600 x 600 DPI)
Figure 5.

170x205mm (600 x 600 DPI)
### Figure 6

175x150mm (600 x 600 DPI)
Figure 7.

170x102mm (600 x 600 DPI)
Table 1. Baseline characteristics.

<table>
<thead>
<tr>
<th></th>
<th>NH-H</th>
<th>OA</th>
<th>OCA</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (patients)</td>
<td>3</td>
<td>10</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>n (nodules)</td>
<td>3</td>
<td>11</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Female, n (%</td>
<td>3 (100%)</td>
<td>7 (70%)</td>
<td>8 (50%)</td>
<td>0.21* ,∥</td>
</tr>
<tr>
<td>Mean age, years (± SD)§</td>
<td>55 ± 17</td>
<td>60 ± 12</td>
<td>54 ± 12</td>
<td>0.53† ,∥</td>
</tr>
<tr>
<td>Median nodule size on</td>
<td>35 (15-35)</td>
<td>25 (17-40)</td>
<td>38 (21-50)</td>
<td>0.41‡</td>
</tr>
<tr>
<td>histopathology, mm (IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bethesda classification</td>
<td>-</td>
<td>-</td>
<td>1 (6%)</td>
<td>0.80*</td>
</tr>
<tr>
<td>III</td>
<td>3 (100%)</td>
<td>7 (64%)</td>
<td>9 (56%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>1 (13%)</td>
<td>2 (13%)</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>3 (27%)</td>
<td>4 (25%)</td>
<td></td>
</tr>
<tr>
<td>Molecular testing</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>on cytology</td>
<td>3 (100%)</td>
<td>3 (27%)</td>
<td>6 (37.5%)</td>
<td>0.07*</td>
</tr>
<tr>
<td>on histopathology</td>
<td>0 (0%)</td>
<td>8 (73%)</td>
<td>10 (62.5%)</td>
<td></td>
</tr>
</tbody>
</table>

*: Pearson’s chi-squared test. †: one-way ANOVA. ‡: Kruskall Wallis test. §: mean age at the time of the CNA-LOH analysis. ∥: calculated on total number of patients. OA, oncocytic thyroid adenoma; OCA, oncocytic thyroid carcinoma; IQR, interquartile range; SD, standard deviation; NH-H, nodular hyperplasia with oncocytic cell metaplasia.
Table 2. Clinical, histopathological and GWLOH data per included nodule (n=30)

<table>
<thead>
<tr>
<th>no.</th>
<th>Sex</th>
<th>Age</th>
<th>Lesion size (mm)</th>
<th>Histopathology</th>
<th>FU status</th>
<th>MD material</th>
<th>Tumour cell %</th>
<th>CNA type</th>
<th>no. of chromosomes involved</th>
<th>Heterogeneity</th>
<th>Endoreduplication</th>
<th>Molecular driver (DNA + RNA)</th>
<th>Molecular diagnosis</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>74</td>
<td>15</td>
<td>NH-H</td>
<td>5 NED</td>
<td>cytology</td>
<td>≥90%</td>
<td>no CNA</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>39</td>
<td>35</td>
<td>NH-H</td>
<td>8 NED</td>
<td>cytology</td>
<td>≥30%</td>
<td>no CNA</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>54</td>
<td>40</td>
<td>NH-H</td>
<td>13 NED</td>
<td>cytology</td>
<td>≥30%</td>
<td>no CNA</td>
<td>BRAF non-v600E, c.1803A&gt;T</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>76</td>
<td>27</td>
<td>OA</td>
<td>0 NED</td>
<td>histology</td>
<td>≥50%</td>
<td>no CNA</td>
<td>EIF1AX, c.338-1G&gt;C</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>66</td>
<td>19</td>
<td>OA</td>
<td>7 NED</td>
<td>cytology</td>
<td>≥90%</td>
<td>RCI type</td>
<td>EIF1AX, c.338-2A&gt;T</td>
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<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>55</td>
<td>49</td>
<td>OA</td>
<td>5 NED</td>
<td>histology</td>
<td>≥80%</td>
<td>RCI type</td>
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<td>none</td>
<td>none</td>
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</tr>
<tr>
<td>7</td>
<td>F</td>
<td>48</td>
<td>17</td>
<td>OA</td>
<td>1 NED</td>
<td>cytology</td>
<td>≥30%</td>
<td>RCI type</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>62</td>
<td>40</td>
<td>OA</td>
<td>2 NED</td>
<td>cytology</td>
<td>≥30%</td>
<td>RCI type</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>49</td>
<td>58</td>
<td>OA</td>
<td>? ?</td>
<td>histology</td>
<td>≥50%</td>
<td>RCI type</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>76</td>
<td>25</td>
<td>OA</td>
<td>? ?</td>
<td>histology</td>
<td>≥70%</td>
<td>RCI type</td>
<td>CDH1, c.220C&gt;T</td>
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<td>none</td>
<td>none</td>
<td>benign</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>55</td>
<td>14</td>
<td>OA</td>
<td>6 NED</td>
<td>histology</td>
<td>≥50%</td>
<td>GH type</td>
<td>3 / 23</td>
<td>yes</td>
<td>no</td>
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<td>12</td>
<td>M</td>
<td>44</td>
<td>25</td>
<td>OA</td>
<td>? ?</td>
<td>histology</td>
<td>≥70%</td>
<td>GH type</td>
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<td>no</td>
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</tr>
<tr>
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<td>histology</td>
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<td>GH type</td>
<td>4 / 23</td>
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<td>14</td>
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<td>3</td>
<td>OA</td>
<td>? ?</td>
<td>histology</td>
<td>≥70%</td>
<td>GH type</td>
<td>10 / 23</td>
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<td>no</td>
<td>none</td>
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<td>Age</td>
<td>Stage</td>
<td>Histology</td>
<td>Cytology</td>
<td>GH Type</td>
<td>CNA</td>
<td>Metastases</td>
<td>Outcome</td>
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<td>histology</td>
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<td>no CNA</td>
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<td>?</td>
<td>?</td>
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<td>none</td>
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<td>NED</td>
<td>histology</td>
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<td>?</td>
<td>histology</td>
<td>≥80%</td>
<td>GH type</td>
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<td>6</td>
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<td>NED</td>
<td>cytology</td>
<td>≥30%</td>
<td>GH type</td>
<td>8 / 23</td>
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<td>no</td>
<td>None</td>
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<td>14</td>
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<td>NED</td>
<td>histology</td>
<td>≥80%</td>
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<td>50</td>
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<td>NED</td>
<td>cytology</td>
<td>≥50%</td>
<td>GH type</td>
<td>12 / 23</td>
<td>no</td>
<td>possible</td>
<td>TERT, c.-124C&gt;T</td>
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<td>mi-OCA, pT2N1bM1, with pulmonary and renal metastases</td>
<td>AWD</td>
<td>histology</td>
<td>≥70%</td>
<td>GH type</td>
<td>13 / 23</td>
<td>yes, limited</td>
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<td>24</td>
<td>F</td>
<td>51</td>
<td>44</td>
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<td>AWD</td>
<td>cytology</td>
<td>≥50%</td>
<td>GH type</td>
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<td>histology</td>
<td>≥60%</td>
<td>GH type</td>
<td>15 / 23</td>
<td>yes, limited</td>
<td>yes</td>
<td>none</td>
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<td>1</td>
<td>AWD</td>
<td>histology</td>
<td>≥80%</td>
<td>GH type</td>
<td>15 / 23</td>
<td>no</td>
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<td>27</td>
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<td>73</td>
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<td>?</td>
<td>?</td>
<td>core biopsy</td>
<td>≥70%</td>
<td>GH type</td>
<td>17 / 23</td>
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<tr>
<td>28</td>
<td>M</td>
<td>46</td>
<td>80</td>
<td>wi-OCA, pT3N0M1, with extensive vascular invasion, pulmonary and osseous metastases</td>
<td>20</td>
<td>AWD</td>
<td>cytology</td>
<td>≥30%</td>
<td>GH type</td>
<td>8 / 23</td>
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<td>29</td>
<td>M</td>
<td>61</td>
<td>85</td>
<td>wi-OCA, pT3N1bM1, with extensive lnn, pulmonary and osseous metastases</td>
<td>17</td>
<td>AWD</td>
<td>cytology</td>
<td>≥80%</td>
<td>GH type</td>
<td>17 / 23</td>
<td>yes</td>
<td>possible</td>
<td>none</td>
</tr>
<tr>
<td>30</td>
<td>M</td>
<td>49</td>
<td>40</td>
<td>wi-OCA, pT3N1bM1, with extensive lnn, pulmonary, and osseous metastases</td>
<td>20</td>
<td>DOD</td>
<td>histology</td>
<td>≥50%</td>
<td>GH type</td>
<td>18 / 23</td>
<td>no</td>
<td>possible</td>
<td>TP53, c.1010_1011dupGC</td>
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*: nodules 12 and 13 are a left- and right-sided nodule in the same patient. Although CNA patterns appear similar in both nodules, further analysis of the SNP profiles demonstrated that the GH type alterations appeared in different alleles on chromosomes 13 and 22 of the two nodules. The lesions were therefore considered of different clonal origin and both included in the current study. ?, follow-up status of patient is unknown. AWD, alive with disease. CHS, cancer hotspot. CNA, copy number alterations. DOD, died of disease-related causes. F, female. FU, follow-up duration in months, measured from date of primary thyroid surgery until date of latest follow-up visit. GH type, genome haploidization type. OA, oncocytic thyroid adenoma. M, male. mi-OCA, minimally invasive oncocytic thyroid carcinoma. NED, no evidence of disease. NH-H, nodular hyperplasia with oncocytic cell metaplasia. RCI type, reciprocal chromosomal imbalance type. wi-OCA, widely invasive oncocytic thyroid carcinoma.
**Box 1** Consideration points for CNA-LOH analysis in daily clinical practice.

- All assessments should be performed by an experienced thyroid pathologist.
- Confirm the presence of true oncocyctic cells in the cytological or histopathological sample by microscopic assessment (Lloyd, *et al.* 2017).
- Consider the tumor cell percentage of the tested sample. In case of a suboptimal tumor cell percentage, i.e., 30-50% in the case of testing on cytology, assessing the possible presence of endoreduplication may be more difficult.
- Four main CNA patterns are distinguished:
  - GH type alterations with (suspected) endoreduplication, consistent with genotype AA or a multiple thereof in the affected chromosomes, associated with OCA, progression of disease and worse prognosis; initial total thyroidectomy may be considered instead of diagnostic hemithyroidectomy depended on the clinical patient context.
  - GH type alterations without (suspected) endoreduplication, consistent with genotype A0 in the affected chromosomes. Observed in both OA and OCA, the number of affected chromosomes and presence of heterogenicity define the molecular diagnosis (Figure 2); diagnostic hemithyroidectomy is recommended to obtain a definitive diagnosis.
  - RCI type with chromosomal copy number gains, genotype AAB, foremost associated with benign, biologically indolent disease. Hemithyroidectomy may be considered.
  - No CNA, normal heterozygous pattern.
- The presence of widespread GH type alterations with suspected endoreduplication likely indicates a biologically more aggressive tumor, even in OCN without signs of capsular or vascular invasion (i.e., morphological OA). (Re-)consideration of a malignant molecular diagnosis is warranted.
- The results of the CNA-LOH analysis should be interpreted alongside the results of somatic mutation and fusion analysis. No CNA are found in nodular hyperplasia with oncocyctic cell metaplasia and part of the OA. The absence of CNA alone does not exclude malignancy and...
does not justify withholding diagnostic surgery for a Bethesda III or IV nodule with cytology suspicious for OCN. If no CNA and no somatic mutations or fusions are detected, however, withholding diagnostic surgery may be considered oncologically safe.

- The results of the CNA-LOH analysis should be interpreted in context of other clinicopathological characteristics, including nodule size.
A clinically applicable molecular classification of oncocyic cell thyroid nodules

Elizabeth J. de Koster, Willem E. Corver, Lioe-Fee de Geus-Oei, Wim J.G. Oyen, Dina Ruano, Abbey Schepers, Marieke Snel, Tom van Wezel, Dennis Vriens, Hans Morreau

Supplementary Data
NGS Somatic mutation analysis

The NGS somatic mutation analysis was performed by GenomeScan BV, on the Ion Torrent GeneStudio™ S5 platform (GenomeScan BV, Leiden, The Netherlands), using the custom Ampliseq™ Cancer Hotspot v6 panel or the custom Ampliseq™ NGS ENDO32 v1 panel (Thermo Fisher Scientific, Waltham, MA, USA), according to the methods as previously described (Aydemirli, et al. 2021; Cohen, et al. 2020; Sibinga Mulder, et al. 2017; van der Tuin, et al. 2019a).

The custom Ampliseq™ Cancer Hotspot v6 panel analyses the following genes: ABL1, AKT1, ALK, APC, ARAF, ATM, BAP1 (exon 3-17 partial), BRAF, CARD11, CD79A, CD79B, CDC73, CDH1, CDK4, CDKN2A, CIC, CSF1R, CTNNB1, CTNNB1, DDR, DICER, EGFR, EIF1AX, ERBB2, ERBB3, ERBB4, ERCC2, ERCC2, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, FOXL2, GNA11, GNAQ, GNAS, H3F3A, H3F3B, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MAP2K1, MAP2K2, MAP2K4, MAP3K1, MDM2, MED12, MET, MLH1, MPL, MUTYH, MYC, MYD88, MyoD1, NKF2-1, NOTCH1, NTRK1, NPM1, NRAS, PDGFRA, PDGFRB, PIK3CA, POLD1, POLE, PTEN, PTK2, PTPN11, RB1, RET, SMAD4, SMARC61, SMO, SRC, STK11, TERT-promoter, TP53 (exon 2-11 partial), and VHL.

The Custom Ampliseq™ NGS ENDO32 v1 panel analyses the coding exons of the following genes: ATP1A1, ATP2B3, ARMC5, CACNA1D, KCNJ5, NF1, TSC1, CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2B, CDKN2C, CDKN2D, CDC73, MEN1, TP53. It also analyses the hotspots in the following genes: BRAF, DICER1, EIF1AX, HRAS, KRAS, NRAS, PIK3CA, PTEN, RET and the TERT-promoter.

NGS fusion analysis

The NGS fusion analysis was performed by GenomeScan BV on the Ion Torrent GeneStudio™ S5 platform (GenomeScan BV, Leiden, The Netherlands), using the Archer FusionPlex CTL Panel v1 or v2 (ArcherDX Inc., Boulder, CO, USA), according to the methods as previously described (Aydemirli, et al. 2021; Cohen, et al. 2020; van der Tuin, et al. 2019a; van der Tuin, et al. 2019b). It uses both
RNA and DNA. Version 1 can detect fusions with the following target genes: ALK, AXL, CCND1, FGFR1, FGFR2, FGFR3, MET, NRG1, NTRK1, NTRK2, NTRK3, PPARG, RAF1, RET, ROS1, and THADA. It can also detect hotspot mutations in BRAF, EGFR, HRAS, KRAS, and NRAS.

Version 2 detects fusions with the following target genes: ALK, AXL, BRAF, CCND1, FGFR1, FGFR2, FGFR3, GLIS1, GLIS3, MET, NRG1, NTRK1, NTRK2, NTRK3, PPARG, RAF1, RET, ROS1, TERT, and THADA. In addition, it detects hotspot mutations in the following genes: AKT1, ALK, BRAF, CTNNB1, DDR2, DICER1, EGFR, EIF1AX, ERBB2, FGFR1, GNAS, HRAS, IDH1, IDH2, KRAS, MAP2K1, NRAS, PIK3CA, RET, and ROS1.
Supplementary Figure 1.
Verification of the CNA patterns observed during CNA-LOH analysis using the GWLOH v2 panel (B and H) using historical cases on which LAIR analysis (A and C-G) was previously performed according to the methods described by Corver et al. (Corver, et al. 2018). Two of the four historical cases are presented in Figure 3 of the main manuscript; here we present two more OCA. A. The SNP array analysis (iCOG and HumanCytoSNP-12, Illumina, Inc., San Diego, CA, USA) of one OCA in a male patient demonstrated CNA with homozygosity and chromosomal losses of chromosomes 1-4, 6, 8-11, 13-17, 19, 21, and 22. Unfortunately, no DNA content analysis by flow cytometry was performed for this case and exact information regarding the DNA index is lacking. Yet, based on the baseline of the SNPs (blue) on the iCOG SNP array, no endoreduplication is suspected. B. On CNA-LOH analysis using the GWLOH panel, this OCA also showed chromosomal losses of the affected chromosomes relative to the unaffected chromosomes (middle panel, normalized median amplicon read count). The VAF of the GWLOH analysis (bottom panel) of the affected chromosomes showed an SNP imbalance with a pronounced but not an extreme amplitude, consistent with GH type CNA. Based on the GWLOH panel, endoreduplication was considered possible, although the results were not highly suspicious for it. Altogether, results of the LAIR analysis are in accordance with the results of the GWLOH panel. The findings correspond best to GH type CNA without (suspected) endoreduplication.

C. The iCOG SNP array analysis of the other OCA in a male patient of this case showed homozygosity and chromosomal losses of chromosomes 1-4, 6, 8, 9, 11, 14-16, 21, and 22. D. Keratin (FITC) vs. vimentin (APC) fluorescence. Different populations R1 (vimentin-positive, keratin negative) and R2 (vimentin-positive and keratin-positive) can be observed. E. Negative control stained with the secondary reagents and PI only. F. Overlay of population R1 and R2. Note that the major $G_0G_1$-peak of the histogram is painted red and left to minor green $G_0G_1$-peak of R1, the DNA-diploid internal reference. This is indicative for loss of DNA. G. The DNA histogram of R2, the keratin-positive, vimentin-positive population showed to be bi-modal due to two cycling populations. In this case, the second $G_0G_1$ peak is significantly lower than that of the first $G_0G_1$ peak. The MFI of the R1 $G_0G_1$-population was used to accurately calculate the DNA index of the two $G_0G_1$ peaks of R2. The DNA indices were 0.6 and 1.1, respectively thus showing loss of DNA and near-haploidy and
Endoreduplication. On the results of the GWLOH panel, chromosomal losses of the affected chromosomes relative to the unaffected chromosomes were observed (middle panel, normalized median amplicon read count), corresponding to GH type CNA. The SNP imbalances (bottom panel) showed high but noisy amplitudes. Endoreduplication was deemed possible, but assessment was difficult. The GWLOH results of this case illustrate that assessing the presence of endoreduplication using GWLOH analysis may be complex, especially in case of limitations regarding specimen quality and/or tumor cell percentage, but also in case of intra-tumor heterogeneity. The tumor cell percentage of the tested sample should always be considered when assessing the possible presence of endoreduplication. Altogether, for this OCA, the CNA pattern observed on the GWLOH panel was consistent with the findings of the LAIR analysis, too.

488 or 633, laser wavelength used for excitation. 530/30, band pass filter used to collect FITC fluorescence (green). 670/14, band pass filter used to collect APC fluorescence (infra-red). >610/20, long pass filter used to collect PI fluorescence (deep-red). APC, allophycocyanin. CNA, copy number alterations. CNA-LOH, copy number alterations and loss of heterozygosity. FITC, fluorescein isothiocyanate. GH type, genome haploidization type. GWLOH, genome-wide loss of heterozygosity. OCA, oncocytic thyroid carcinoma. LAIR, lesser-allele intensity-ratio. MFI, median fluorescence intensity. SNP, single nucleotide polymorphism. VAF, variant allele frequency.
References


