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Molecular evidence in support of the neoplastic and precursor nature of microglandular adenosis

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Abstract

Aims: Microglandular adenosis (MGA) is a proliferative breast lesion, which has been proposed to be a potential precursor of triple-negative breast cancers. The aims of this study were to determine whether MGAs harbour genetic alterations and if any such genetic aberrations found in MGAs are similar to those found in matched invasive carcinomas.

Methods and results: Twelve cases of MGA and/or atypical MGA (AMGA), 10 of which were associated with invasive carcinoma, were evaluated. Immunohistochemical profiling revealed that all invasive carcinomas were of triple-negative phenotype and expressed S100, cytokeratins 8/18 and 'basal' markers. The morphologically distinct components of each case (MGA, AMGA and/or invasive carcinoma) were microdissected and subjected to microarray comparative genomic hybridization. Apart from three typical MGAs, all samples harboured genetic alterations. The percentage of the genome affected by copy number aberrations in MGA/AMGA ranged from 0.5 to 61.9%, indicating varying levels of genetic instability. In three cases, MGA/AMGA displayed copy number aberrations similar to those found in matched invasive components, providing strong circumstantial evidence that MGA may constitute the substrate for the invasive carcinoma development.

Conclusions: Our results support the contention that MGA can be a clonal lesion and non-obligate precursor of triple-negative breast cancer.

Abbreviations:

AMGA: atypical MGA

CGH: comparative genomic hybridization

CHORI: chromosome re-array collection

CK: cytokeratin

ER: oestrogen receptor

FFPE: formalin-fixed paraffin-embedded

HER2: human epidermal growth factor receptor 2

MGA: microglandular adenosis

PR: progesterone receptor

Introduction

Microglandular adenosis (MGA) is a rare proliferative lesion of the breast. Although currently classified as adenosis, MGA differs substantially at the histological and immunophenotypic levels from other lesions classified as 'adenosis'.1 Histologically, MGA is characterized by small glands with open lumina, lined by a single layer of cuboidal-to-flattened epithelial cells arranged in a rather infiltrative pattern within a fibro-fatty stroma. Unlike other forms of adenosis, MGA is not composed of a dual cell population and lacks a myoepithelial cell layer. Furthermore, the cells of MGA have a typical immunophenotype; that is, MGA cells lack oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression (i.e. triple-negative phenotype), and express S100 protein.[1–4]

There is burgeoning evidence to suggest that MGA is not merely hyperplastic, but may represent a neoplastic clonal lesion and a non-obligate precursor of a subset of breast cancers.2–8 Atypical forms of MGA (AMGA) and frequent association with invasive carcinomas which tend to be of high histological grade have been documented in the literature.2–8 In keeping with this, invasive tumours often recapitulate the morphology of associated MGA and AMGA components, such as clear cell features, cytoplasmic acinic cell-like granules and secretory activity.[2–6]

Recent studies have catalogued in part the molecular features of the spectrum of MGA-related lesions and demonstrated that MGA, AMGA and invasive tumours arising in association with MGA share strikingly similar immunophenotypic and genetic features.[2,3,7] The great majority, if not all, of MGA-associated invasive cancers are reported to be of triple-negative phenotype and strongly express S100.[2,3] Furthermore, expression of basal-like markers such as high molecular weight cytokeratins (CKs), and epidermal growth factor receptor (EGFR) is also observed across this spectrum of lesions.[2,3] We[2] and others[7] have undertaken whole genomic analysis with comparative genomic hybridization (CGH) of cases composed of MGA, AMGA and associated invasive cancers and demonstrated that at least some MGA display DNA copy number alterations detectable by CGH and that the distinct components of a given case share the same pattern of genetic changes. It has therefore been postulated that MGA is a clonal lesion with a potential to progress to high-grade triple-negative invasive cancers. This has led us to suggest that the term 'microglandular adenoma' would be more appropriate and reflective of the underlying tumour biology.[2]

It should be noted, however, that all but one of the cases were subjected to CGH analysis after whole genome amplification, an approach that inevitably leads to bias in the analysis of copy number aberrations.9 Owing to the paucity of high-resolution genetic data to investigate the similarities between MGA and matched invasive cancers (i.e. carcinomas arising in the same breast adjacent to MGA), we studied a series of 12 cases composed of MGA and/or AMGA, 10 of which were associated with invasive

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breast carcinomas. In each lesion, morphologically distinct components were microdissected and subjected to high-resolution microarray-based CGH (aCGH). Genetic changes were detected in the majority of the samples analysed and matched MGA, AMGA and invasive carcinoma samples displayed similar genomic profiles, providing additional molecular evidence for the progression of MGA and AMGA to invasive carcinoma. In addition, our results revealed the heterogeneity of MGAs at the genetic level.

Material and methods

Cases

Twelve cases composed of MGA and/or AMGA, of which 10 displayed an associated-invasive component, were included in this study (Table 1): a previously reported index case from Leicester University Hospital NHS Trust, Leicester, UK,[2] seven cases retrieved from the archives of the MD Anderson Cancer Center, Texas, USA and four additional cases, one from the Royal Marsden Hospital, London, UK, two from the Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands and one from Ospedale A. Businco, Cagliari, Italy. The morphological and immunohistochemical characteristics of eight cases have been reported elsewhere.[2,3] Ethical approval was obtained from local ethical committees.

Table 1. Summary of 12 cases composed of MGA and/or AMGA and/or associated invasive carcinomas

Case	Origin	Invasive component histological type	Microdissected components/DNA extracted	aCGH analysis	Reference
Index	University Leicester Hospital NHS Trust	High-grade IDC-NST	MGA, AMGA, invasive	Yes*	2
5	MD Anderson Cancer Center	Acinic-like/matrix-producing/sarcomatoid	AMGA, invasive	Yes	3
6	MD Anderson Cancer Center	Matrix-producing	AMGA, invasive	NP	3
7	MD Anderson Cancer Center	Adenoid cystic/matrix-producing	MGA	NP	3
8	MD Anderson Cancer Center	Matrix-producing	MGA, invasive	Yes	3
9	MD Anderson Cancer Center	Acinic-like	MGA, bone MTX	NP	3
10	MD Anderson Cancer Center	Acinic-like/sarcomatoid	AMGA	Yes	3
11	MD Anderson Cancer Center	High-grade IDC-NST	MGA, AMGA, invasive	Yes	3
12	Royal Marsden Hospital	Matrix-producing	MGA, invasive	Yes	Not previously described
13	Netherlands Cancer Institute	High grade IDC-NST	MGA, AMGA, DCIS, invasive	Yes	Not previously described
14	Netherlands Cancer Institute	No invasive component	MGA, AMGA	Yes	Not previously described
15	Ospedale A. Businco	No invasive component	MGA	Yes	Not previously describe

aCGH, Microarray-based comparative genomic hybridization; AMGA, atypical microglandular adenosis; IDC-NST, invasive ductal carcinoma of no special type; MGA, microglandular adenosis; MTX, metastasis; NP, not performed.

*aCGH analysis described previously.

Haematoxylin and eosin-stained sections of each case were reviewed by at least three pathologists (F.C.G., M.L.-T., A.G. and/or J.S.R.-F.), and the distinct components of each case (i.e. MGA, AMGA and invasive carcinoma) were categorized based on previously described criteria.[3,4,8] Briefly, MGA was defined as a lesion composed of small round glandular structures with open lumina distributed randomly in fibrocollagenous mammary stroma. The glands are formed by a single layer of cuboidal-to-flattened epithelial cells and lack a myoepithelial cell layer. Lesions were classified as AMGA if composed of irregular glands, arranged in a back-to-back pattern without desmoplasia, with mild-to-moderate nuclear pleomorphism, scattered apoptotic and mitotic figures. Lesions with coalescent growth of atypical cells, with associated desmoplastic reaction and/or infiltrating cords and isolated cells surrounded by a desmoplastic reaction were categorized as invasive. The histological characteristics of the invasive carcinoma components were also recorded.

Immunohistochemistry

Immunohistochemical analysis of the MD Anderson (n=7) and index (n=1) cases have been described elsewhere.[2,3] Immunohistochemistry for the new cases (n=4) was performed on 3-µm sections, as described previously (Table S1),[2] using antibodies against ER, PR, HER2, S100, EGFR, low molecular weight CK8/18, high molecular weight CK5/6, 14 and 17 and p63. Sections subjected to immunohistochemistry were analysed by at least three pathologists (F.C.G., M.L.-T., A.G. and/or J.S.R.-F.) and markers were scored as described previously (Table S1).[2,10] In brief, ER and PR were considered positive if >1% of neoplastic cells exhibited nuclear expression. HER2 was scored according to the current American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines.[11] Membranous staining for EGFR in more than 10% of the cells was considered positive as described previously.[3] A lesion was considered positive for the expression of S100 protein if any morphologically unequivocal neoplastic cells displayed nuclear and/or cytoplasmic expression. For CK8/18, CK5/6, CK14 and CK17, a lesion was considered positive if any morphologically unequivocal neoplastic cells displayed cytoplasmic expression.[2] A semi-quantitative system was employed to report the results of the analysis of S100, CK8/18, CK5/6, CK14 and CK17, namely 0, negative; +/−, <10% of neoplastic cells expressing the marker; +, 10 −≤25% of neoplastic cells expressing the marker; ++, >25–50% of neoplastic cells expressing the marker; +++, >50% of neoplastic cells expressing the marker.[2] Immunohistochemical analysis of p63 was

performed to confirm the lack of a myoepithelial cell layer in MGAs and AMGAs; only nuclear staining in cells was considered specific.[2]

Microdissection and DNA extraction

Ten 8-μm tissue sections of cases 12, 13, 14 and 15 and available tissue sections of the MD Anderson cases (median n = 13, range 7–21) were stained with nuclear fast red and distinct components were microdissected separately with a sterile needle under a stereomicroscope (Olympus SZ61, Tokyo, Japan), as described previously,[10] to ensure >70% of purity of cancer cells. DNA was extracted as described previously[10] using the formalin-fixed paraffin-embedded (FFPE) DNA extraction kit (FFPE DNeasy Kit; Qiagen, West Sussex, UK). DNA concentration was measured using PicoGreen® assay, according to the manufacturer's protocol (Invitrogen, Paisley, UK).

Microarray comparative genomic hybridization

The 32K bacterial artificial chromosome re-array collection (CHORI) tiling path aCGH platform was constructed at the Breakthrough Breast Cancer Research Centre, as described previously.[10] This type of bacterial artificial chromosome array platform has been shown to be as robust as, and with comparable resolution to, high-density oligonucleotide arrays.[12–14] DNA labelling, array hybridizations and image acquisition were performed as described previously.[15] Data were pre-processed and analysed using an in-house R script (BACE.R) in r version 2.9.0, as described previously.[10] After filtering polymorphic bacterial artificial chromosomes, a final data set of 31 367 clones with unambiguous mapping information according to the build hg19 of the human genome (http://www.ensembl.org) was smoothed using the circular binary segmentation (cbs) algorithm.[10,16] Copy number changes were categorized as gains, losses or amplifications according to previously validated thresholds for each clone.[15,16] Threshold values were chosen to correspond to three standard deviations of the normal ratios obtained from the filtered clones mapping to chromosomes 1–22, assessed in multiple hybridizations between DNA extracted from a pool of male and female blood donors, as described previously ($\log_2$ ratio of ±0.08). Losses were defined as cbs-smoothed $\log_2$ ratios <0.08; gains as cbs-smoothed $\log_2$ ratios between 0.08 and 0.45, corresponding to approximately three to five copies of the locus; and amplifications as cbs-smoothed $\log_2$ ratios >0.45, corresponding to more than five copies. Hierarchical clustering analysis was performed as described previously,[10] based on categorical aCGH states (i.e. gains, losses and amplifications) and employing Ward's clustering algorithm and Euclidean distance.

Results

Cases and histological analysis

All cases included in this study are summarized in Table 1. Morphological features of all but four (cases 12–15) have been described previously.[2,3] Briefly, all but two cases were composed of MGA and/or AMGA admixed with an invasive carcinoma. Apart from case 12, a clear transition from MGA to AMGA and invasive cells, frequently with similar cytological features, was observed. Case 12 (Figure 1) was composed of a matrix-producing metaplastic carcinoma (Figure 1A, upper left, and 1D) surrounded by typical MGA (Figure 1A, lower right, 1B and 1C). In this case, AMGA was not identified. Case 13 was composed of MGA, AMGA, ductal carcinoma *in situ* (DCIS) (i.e. an intraductal neoplastic proliferation composed of cells with cytological features similar to those of the AMGA areas surrounded by basement membrane and myoepithelial cells) and a high-grade invasive ductal carcinoma of no special type. Case 14 was composed of MGA and AMGA, whereas case 15 was composed only of typical MGA with no evidence of AMGA, DCIS or invasive carcinoma. It should be noted that only case 15 did not produce a clinically or radiologically detectable mass and was an incidental microscopic finding.
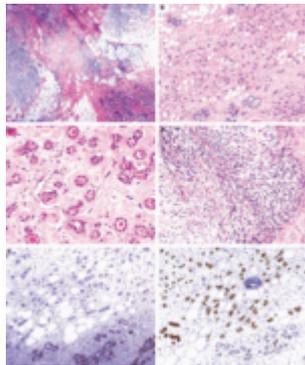

Figure1. Morphological and immunohistochemical features of microglandular adenosis (MGA) and invasive components of case 12. Case 12 was composed of an invasive metaplastic carcinoma (**A,** upper left) surrounded by MGA (**A,** bottom right). MGA was characterized by infiltrative glands with open lumen distributed randomly in a fibrocollagenous stroma (**B**). Those glands were lined by a single layer of cuboidal cells with small and round nuclei, and showed intraluminal secretion (**C**). The invasive component displayed epithelioid cells arranged in a chondroid or myxo-chondroid matrix (**D**). MGA glands lacked myoepithelial cells as highlighted by immunohistochemistry with antibodies raised against p63 (**E**) and displayed strong expression of S100 (**F**).

Multiple histological patterns were detected in the invasive components, including metaplastic carcinomas and the salivary gland analogues acinic cell-like and adenoid cystic subtypes (Table 1, Figure 1D). Although MGA-associated carcinomas are heterogeneous at the morphological level, it should be noted that these histological special types of breast cancer have been shown to be consistently of triple-negative phenotype and classified as basal-like subtype using microarray gene expression profiling and/or immunohistochemical surrogate markers.[10,17–24]

Immunohistochemical analysis

The immunohistochemical features of all cases are summarized in Table 2. As reported previously, the MGA, AMGA and invasive components of the index case and cases [5–11] were negative for ER, PR and HER2, strongly expressed S100 and CK8/18 and showed focal expression of high molecular weight CKs and/or EGFR. Accordingly, the four new cases (12–15) displayed a similar immunophenotype: MGA, AMGA and/or the invasive components were negative for hormone receptors and HER2, and diffusely expressed CK8/18 and S100 protein (Figure 1F). In cases 12, 13 and 14, the lesions also expressed high molecular weight CKs (in case 15, no material was available for additional immunohistochemical analysis). Taken together, all invasive cases described here displayed a triple-negative phenotype, expressed 'basal' markers and would be classified as of basal-like molecular subtype according to a validated immunohistochemical panel.[25]

Table 2. Summary of immunohistochemical features of 12 cases composed of MGA and/or AMGA and/or associated invasive carcinomas

Case	ER	PR	HER2	S100	CK8/18	HMW-CKs	EGFR	Reference
Index	–	–	–	+++	+++	+/–	–	2
5	–	–	–	+++	+++	–	+	3
6	–	–	–	+++	+++	–	+	3
7	–	–	–	+++	+++	–	+	3
8	–	–	–	+++	+++	–	+	3
9	–	–	–	+++	+++	–	+	3
10	–	–	–	+++	+++	–	+	3
11	–	–	–	+++	+++	–	+	3
12	–	–	–	+++	+++	+/–	+	New case
13	–	–	–	+++	+++	–	+	New case
14	–	–	–	+++	+++	–	+	New case
15	–	–	–	+++	+++	NP	NP	New case

–, Negative; +/–, focally positive; +, positive; +++, strongly positive; CK, Cytokeratin; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; HMW-CKs, high molecular weight CKs; NP, not performed; PR, progesterone receptor.

Microarray comparative genomic hybridization analysis

Eight cases yielded DNA of sufficient quality and quantity for aCGH analysis of at least one component (case 5, AMGA and invasive; case 8, MGA and invasive; case 10, AMGA; case 11, MGA, AMGA and invasive; case 12, MGA and invasive; case 13, MGA, AMGA, DCIS and invasive; case 14, MGA and AMGA; case 15, MGA). Results of the whole-genome copy number analysis are summarized in Table 3 and illustrated

in Figures 2–4. Apart from the MGAs of cases 7, 12 and 15, all samples including the typical MGA of cases 11, 13 and 14 (Figures 3 and 4) displayed genetic alterations. Including aCGH data of the index case, all samples analysed displayed on average 20.3% (median 14.95%, range 0.5–61.9%, *n* = 13, Table 3) of the genome harbouring DNA copy number changes, demonstrating that MGAs constitute a heterogeneous group of lesions at the genetic level. While some MGAs and AMGAs displayed a complex genomic profile with gains and losses affecting most of the chromosomes, such as the lesions from cases 5, 8, 13 and 14, others harboured few or no significant copy number alterations (Table 3).

Case	Component	Proportion of the genome with changes (%)	Whole genome Pearson correlation
Index	MGA	9.2	MGA versus AMGA: 0.85 AMGA versus invasive: 0.88 MGA versus invasive: 0.88 *P* < 0.05
Index	AMGA	9.2	
Index	Invasive	9.3	
5	AMGA	29.9	AMGA versus invasive: 0.76 *P* < 0.05
5	Invasive	40.3	
7	MGA	1.1	NS
7	Invasive	6.1	
8	AMGA	46.3	NA
11	MGA	18.2	MGA versus AMGA: 0.77 AMGA versus invasive: 0.77 MGA versus invasive: 0.68 *P* < 0.05
11	AMGA	13.1	
11	Invasive	11.7	
12	MGA	0.5	NS
12	Invasive	61.9	
13	MGA	26.9	MGA versus AMGA: 0.72 MGA versus DCIS: 0.77 MGA versus invasive: 0.67 AMGA versus DCIS: 0.69 AMGA versus invasive: 0.71 DCIS versus invasive: 0.81 *P* < 0.05
13	AMGA	15.0	
13	DCIS	21.1	
13	Invasive	41.0	
14	MGA	22.1	MGA versus AMGA: 0.71 *P* < 0.05
14	AMGA	14.9	
15	MGA	6.0	NA

Table 3. Summary of genomic features of nine cases composed of MGA and/or AMGA and/or associated invasive carcinomas

AMGA, Atypical microglandular adenosis; DCIS, ductal carcinoma *in situ*; MGA, microglandular adenosis; NA, not applicable; NS, not significant.
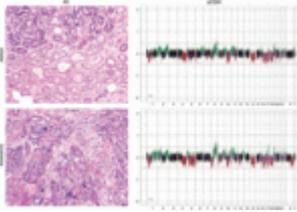
Figure2. Representative micrographs and genome plots of case 5, atypical microglandular adenosis (AMGA) and invasive components. Representative micrograph of the AMGA component characterized by irregular glands, arranged in a back-to-back pattern without desmoplasia, lined by cuboidal cells with mild-to-moderate nuclear pleomorphism and cytoplasmic acinic cell-like granules. Representative micrograph of the invasive component characterized by nests of pleomorphic epithelioid and plump spindle cells; scattered cells with intracytoplasmic acinic cell-like granules are observed. In the genome plots, circular binary segmentation (CBS) $\log_2$ ratios are plotted on the *y*-axis against each clone according to genomic location on the *x*-axis. Bacterial artificial chromosomes (BACs) categorized as displaying genomic gains, amplifications or losses are plotted in green, bright green or red, respectively. aCGH: microarray-based comparative genomic hybridization; MGA: microglandular adenosis.
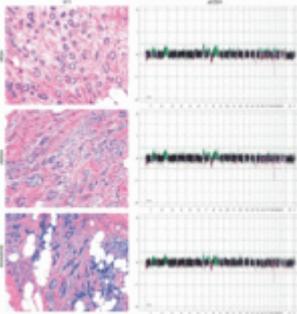

Figure3. Representative micrographs and genome plots of case 11 microglandular adenosis (MGA), atypical microglandular adenosis (AMGA) and invasive components. Representative micrograph of the MGA component, which was composed of small round glandular structures with open lumens distributed randomly in fibrocollagenous mammary stroma. In the AMGA component, the glands are arranged in a back-to-back fashion and are lined by cells more atypical with mild-to-moderate pleomorphic nuclei. Note that in the invasive ductal carcinoma of no special type component, stromal desmoplasia is observed. In genome plots, circular binary segmentation (CBS) log2 ratios are plotted on the *y*-axis against each clone according to genomic location on the *x*-axis. Bacterial artificial chromosomes (BACs) categorized as displaying genomic gains, amplifications or losses are plotted in green, bright green or red, respectively. aCGH, microarray-based comparative genomic hybridization.
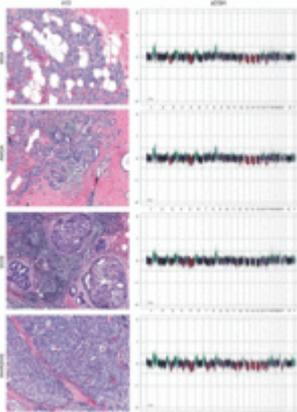

Figure 4. Representative micrographs and genome plots of case 13 microglandular adenosis (MGA), atypical microglandular adenosis (AMGA), ductal carcinoma *in situ* (DCIS) and invasive components. Representative micrograph of the MGA component, which was composed of small round glandular structures distributed randomly in a fibrocollagenous stroma. In the AMGA component, the glands are enlarged and irregular and are lined by atypical cells with moderately pleomorphic nuclei. Note the presence of a lymphocytic infiltrate. The DCIS component was in the form of an intraductal proliferation of cells with similar cytological characteristics arranged in solid and cribriform patterns, and surrounded by both myoepithelial cells and basement membrane. The high-grade invasive ductal carcinoma of no special type component displayed a solid pattern, with focal tubule formation. In genome plots, circular binary segmentation (CBS) log2 ratios are plotted on the *y*-axis against each clone according to genomic location on the *x*-axis. Bacterial artificial chromosomes (BACs) categorized as displaying genomic gains, amplifications or losses are plotted in green, bright green or red, respectively. aCGH, microarray-based comparative genomic hybridization.

When MGA, AMGA and invasive lesions were considered separately, an increase in the percentage of the genome harbouring changes was observed in the progression from MGA to AMGA and to invasion. MGA samples displayed an average of 12.0% (median 9.2%, range 0.5–26.9%) of the genome with changes; AMGA, 21.4% (median 14.95%, range 9.2–46.3%); and invasive lesions, 28.4% (median 26.0%, range 9.3–61.9%). The levels of genetic instability and the pattern of genetic aberrations, however, tended to exhibit greater concordance between matched samples than across samples of the same morphological category (Figure 5). In cases 5, 11 and 13, the genomic profiles of MGA and/or AMGA and matched invasive

components were available for comparison (Figures 2–4). Consistent with the observations derived from the analysis of the levels of genetic instability, hierarchical clustering revealed that morphologically distinct lesions from the same tumour clustered together preferentially, rather than with morphologically comparable components from other tumours (Figure 5). Despite the small sample size, these observations provide another line of evidence to suggest that matched MGA, AMGA and invasive carcinomas developing in the same breast were more similar to one another than lesions of the same category from separate cases. In agreement with the results of our previously published index case,[2] MGA and/or AMGA components displayed copy number aberrations similar and correlated significantly with those found in matched invasive components (Pearson's $r \geq 0.67$, $P < 0.05$; Table 3), demonstrating that the distinct components from each case were clonally related and that MGA may constitute the substrate for the development of the invasive carcinoma. In cases 5 and 13, the invasive component harboured genetic aberrations in addition to those found in the MGA or AMGA, such as gains of 1q and losses of 5q, and gains of 7p and losses of 7q, respectively (Figures 2 and 4).
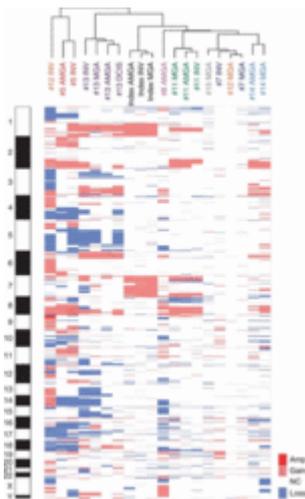

Figure 5. Unsupervised hierarchical cluster analysis of nine cases composed of microglandular adenosis (MGA) and/or atypical MGA (AMGA) and/or associated invasive carcinomas (*n*=20 samples). Dendrogram and heatmap generated by hierarchical clustering (Ward's clustering algorithm and Euclidean distance) using microarray-based comparative genomic hybridization (aCGH) categorical data derived from 31 367 bacterial artificial chromosomes (BACs), including nine cases/20 samples [seven MGAs, six AMGAs, one ductal carcinoma *in situ* (DCIS) and six associated invasive carcinomas]. Rows: cbs-log$_2$ ratios categorized as gains (red), losses (blue) and no change (white) for each BAC clone in genomic order. Note that distinct components of matched samples cluster together preferentially rather than with samples of the same morphological category. Amp: amplification; INV: invasive carcinoma; NC: no change.

The MGA component of cases 7, 12 and 15 displayed a flat profile, with no significant copy number changes. In case 7, the genomic profile of the invasive component was concordant, showing few copy number changes, suggesting low levels of genetic instability in the entire tumour. Conversely, in case 12, the invasive component harboured the highest proportion of the genome with genetic alterations. These findings are in agreement with those of a previous report[7] describing lack of copy number aberrations in a subset of MGAs and AMGAs.

Despite the modest sample size, we performed an exploratory analysis of the recurrent gains, losses and amplifications present in the MGA and AMGA samples. In the MGAs (*n*=7), recurrent changes (present in ≥3 cases) included gains of 1q, 2q, 7p, 7q and 8q, and losses of 1p, 8p, 14q, 16q and 17q (Figure 6A). In the AMGA samples (*n*=6), consistent with their more complex and atypical histological features,

additional recurrent changes were observed, such as gains of 6p and losses of 10q (Figure 6B). After exclusion of regions of copy number polymorphism (http://projects.tcag.ca/variation/), no recurrent amplifications were detected. The region on 8q21.2 which was amplified in two samples of AMGA (cases 5 and 10) is a known germline copy number polymorphism (http://projects.tcag.ca/variation/). The lack of recurrent amplifications should not come as a surprise, given that triple-negative and basal-like invasive breast cancers are known to display few recurrent focal high-level gene amplifications.[15,24,26–28]
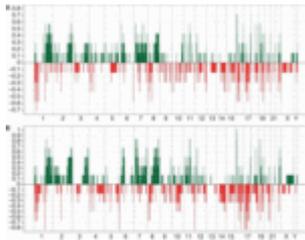

Figure 6. Frequency plots of copy number changes in microglandular adenosis (MGA) (**A,**n = 7) and atypical MGA (AMGA) (**B,**n = 6) samples. In frequency plots, the proportion of tumours in which each clone is gained (green bars) or lost (red bars) is plotted (y-axis) for each bacterial artificial chromosome (BAC) clone according to genomic location (x-axis).

Discussion

We describe here an aCGH-based analysis of a series of cases encompassing MGA, AMGA and MGA-associated invasive carcinomas, all exhibiting a triple-negative phenotype with expression of 'basal' markers. Our results corroborate previous findings[3,7] and confirm that a subset of MGAs are clonal lesions that harbour genetic aberrations. The genetic aberrations found in the MGA/AMGA were similar to those found in samples of adjacent invasive breast cancers. As in our index case,[2] the distinct matched components of four cases displayed genetic aberrations with similar breakpoints. Notably, in cases 5 and 13, the acquisition of additional genetic alterations was apparent in the progression from AMGA to the invasive component, consistent with a clonal evolution process. Taken together, our results and those reported by Shin *et al.*[7] provide strong circumstantial evidence to suggest that MGA is a non-obligate direct precursor of triple-negative breast cancers. Our results also demonstrate, however, that MGAs comprise a genetically heterogeneous group of lesions; while some MGAs have relatively complex patterns of copy number aberrations similar to those found in high-grade triple-negative breast cancers,[15,29–33] others lack any copy number aberrations.

Our findings are consistent with our recently revised hypothetical multistep model of breast cancer evolution.[34] In this model breast cancer development and progression would follow two main molecular pathways according to the expression of ER and ER-regulated genes. MGA would constitute the first morphologically identifiable non-obligate precursors that may give rise to triple-negative invasive carcinomas.[20,22,34,35] It should be noted, however, that not all triple-negative invasive carcinomas may stem from MGA; in fact, it is entirely plausible that the majority of these cancers may evolve without an MGA stage. Unlike precursors of low grade ER-

positive tumours, which have been grouped under the term 'low-grade breast neoplasia family' and are characterized consistently by concurrent gains of 1q and losses of 16q,[34,36–38] MGAs are more heterogeneous at the genetic level.

Consistent with the results described by Shin *et al.*,[7] in this study we demonstrate that the majority of MGAs associated with invasive cancer harbour copy number aberrations and recurrent gains of 1q, 2q and 8q and losses of 14q. Our data suggest that gains of 6p and losses of 10q are more prevalent in AMGA than in MGA. One could hypothesize that activation or inactivation of genes mapping to these genetic regions may be responsible for phenotypic progression. Notably, 6p gains have been associated previously with triple-negative/basal-like invasive breast cancers.[15,26–28] Gains of 8q, which were found in nine of 13 (69%) MGA/AMGA samples, may be of particular relevance to MGA/AMGA development. Contrary to the results of Shin *et al.*,[7] who described *MYC* gene (8q24.21) amplification in three of 13 (23%) cases, we have only detected low-level gains of *MYC* locus in eight of 13 (62%) MGA/AMGA samples and no focal, high-level *MYC* gene amplifications, using extensively validated aCGH platform and thresholds.[15,30,33,39] These differences may be attributable to the small sample sizes in both studies or differences in the methodologies employed to detect *MYC* gene amplification. It is noteworthy that, owing to the small sample sizes of the present study and that by Shin *et al.*,[7] these results should be perceived as hypothesis-generating. Further studies in larger cohorts of MGA/AMGA and matched invasive carcinomas are warranted to confirm these findings and define the molecular driver(s) of MGA and of the progression to an atypical and invasive phenotype.

Based on the transcriptomic similarities between basal/myoepithelial cells of the breast and basal-like and triple-negative breast cancers,[35,40–44] it was hypothesized originally that basal-like and triple-negative cancers would originate from basal/myoepithelial cells.[40–42,44–46] The phenotypic characteristics of basal-like and triple-negative breast cancers, however, are not entirely consistent with those of basal/myoepithelial cells, as these cancers express 'luminal' keratins (e.g. CK8/18) and lack expression of myoid markers (e.g. calponin, smooth muscle actin, smooth muscle myosin heavy chain) and p63, proteins usually found in basal/myoepithelial cells of the breast.[24,47] In fact, recent studies have called into question the notion that basal-like and triple-negative breast cancers would originate from basal/myoepithelial cells,[48,49] and provided direct evidence to demonstrate that these tumours stem from luminal progenitor cells, which express both 'luminal' and 'basal' keratins, EGFR and c-KIT, and lack expression of myoid markers and p63.[48] Consistent with the observations derived from the analysis of basal-like and triple-negative cancers, our results demonstrate that the phenotypic characteristics of MGA/AMGA would also be consistent with those reported for normal breast luminal

progenitor cells (e.g. expression of both 'luminal' and high molecular weight CKs and EGFR; lack of expression of p63).[48]

Of the seven MGA samples described here, copy number alterations were detected by aCGH in four, suggesting that the majority of typical MGAs are clonal neoplastic lesions before the development of morphologically recognizable atypia. These findings are in agreement with the data by Shin et al.,[7] who analysed three pure MGA samples (i.e. not associated with atypia and invasive tumour), one of which displayed numerous chromosomal gains and losses, and provide support to our earlier suggestion of the use of the term 'microglandular adenoma'.[2] It should be noted, however, that the only case not associated with atypical and invasive cancer analysed in this study (case 15) failed to show significant copy number aberrations. In addition, two of the MGAs associated with invasive cancers (cases 7 and 12) lacked any chromosomal aberrations. These observations suggest that MGA comprises a genetically heterogeneous group of lesions which may constitute a convergent phenotype.[50] One of the possible explanations for this diversity is that distinct molecular subgroups of MGA may harbour impairment of distinct mechanisms of DNA repair. It is also plausible that this genetic heterogeneity would perhaps be reflected in the invasive counterparts of MGA, in keeping with the known molecular heterogeneity of triple-negative breast cancers.[20,24] Other explanations are that either a subgroup of MGAs is not driven by genetic aberrations but by epigenetic changes or that the subgroup of MGAs lacking chromosomal aberrations harbour genetic alterations which cannot be detected by aCGH (e.g. point mutations or structural rearrangements). Further analyses based on massively parallel sequencing[51,52] to determine the mutational repertoire of MGAs, AMGAs and matched carcinomas are warranted.

From a clinical standpoint, it remains to be determined how often MGA progresses to triple-negative invasive cancer and the proportion of triple-negative cancers that originate from MGAs. Given that MGA and AMGA lack a myopepithelial cell layer, may display high proliferative activity and, as illustrated here, may harbour considerable genetic instability, progression from MGA to an invasive phenotype may constitute a rapid and frequent event. It is also plausible that MGA and AMGA lesions may be quickly overgrown by their invasive counterparts and therefore not identified, in particular in cases of high-grade triple-negative breast cancers, which display remarkably high levels of proliferation.[24] Due to the relative rarity of MGA and AMGA and the paucity of observational studies of these lesions, the actual rate of progression of MGA/AMGA to invasive breast cancer is yet to be defined. Importantly the available data reporting rates of association with carcinoma as high as 64% are likely to be confounded by referral bias.[3] Nevertheless, data from molecular analyses (described here and elsewhere[2,7]) and observational studies of

MGA[4–6,8] provide a basis for recommending complete resection of all MGAs and AMGAs with clear margins when diagnosed on core needle-biopsy, and thorough examination of samples with MGA and/or AMGA to rule out the present of a concurrent invasive carcinoma. One may argue that the majority of MGAs with no atypia identified as incidental microscopic findings in biopsies taken for other reasons are likely to display low levels of genetic instability, as observed in case 15, and therefore the probability of progression may not be sufficient to trigger additional therapeutic interventions. Further follow-up studies are required to determine the optimal management of patients with MGAs and AMGAs.

In conclusion, we have demonstrated that MGA are genetically heterogeneous and at least some are clonal neoplastic lesions displaying chromosomal aberrations. Concordant genetic aberrations were detected in matched MGA, AMGA and invasive triple-negative breast carcinomas. Therefore, the data presented in this study lend further credence to the contention that MGA is a non-obligate direct precursor of a subgroup of triple-negative breast cancers. Further molecular studies are warranted to identify the molecular drivers of MGA and of the progression from MGA/AMGA to invasive triple-negative breast cancers.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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