CLINICAL STUDY

Differential developmental expression of transcription factors GATA-4 and GATA-6, their cofactor FOG-2 and downstream target genes in testicular carcinoma *in situ* and germ cell tumors

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Abstract

Objective: Testicular germ cell cancer is the most common malignancy among young males. The pre-invasive precursor, carcinoma *in situ* testis (CIS), presumably originates from arrested and transformed fetal gonocytes. Given that GATA transcription factors have essential roles in embryonic and testicular development, we explored the expression of GATA-4, GATA-6, cofactor friend of GATA (FOG)-2, and downstream target genes during human testis development and addressed the question whether changes in this pathway may contribute to germ cell neoplasms.

Methods: Fetal testis, testicular CIS, and overt tumor samples were analyzed by immunohistochemistry for GATA-4, GATA-6, FOG-2, steroidogenic factor 1 (NR5A1/SF1), anti-Müllerian hormone/Müllerian-inhibiting substance (AMH), and inhibin- α (INH α).

Results: GATA-4 was not expressed in normal germ cells, except for a subset of gonocytes at the 15th gestational week. The CIS cells expressed GATA-4 and GATA-6 heterogeneously, whereas most of the CIS cells expressed GATA-4 cofactor FOG-2. GATA target gene *SF-1* was expressed heterogeneously in CIS cells, whereas INH α and AMH were mostly negative. Seminomas and yolk sac tumors were positive for GATA-4 and GATA-6, but mostly negative for FOG-2 and the GATA target genes. In contrast, pluripotent embryonal carcinomas and choriocarcinomas were GATA-4 and GATA-6 negative.

Conclusions: Differential expression of the GATA-4 target genes suggested cell-specific functions of GATA-4 in the germ and somatic cells. The GATA-4 expression in early fetal gonocytes, CIS, and seminoma cells but the absence in more mature germ cells is consistent with the early fetal origin of CIS cells and suggests that GATA-4 is involved in early germ cell differentiation.

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Introduction

Six GATA factors (GATA-1 to GATA-6) are evolutionary conserved transcription factors that bind the consensus motif WGATAR in the regulatory regions of various genes. GATA-4 has important roles in the fetal development of the gonads (1–4). Several GATA-4 target genes are essential in gonadal differentiation steroidogenic factor 1 (*SF1*, *SRY*, *SOX9*) and hormone regulation anti-Müllerian hormone (*AMH*, inhibin- α (*INH- α*)) (3, 5–8). During fetal testis development, GATA-4 and GATA-6 are expressed in mouse and human Sertoli and Leydig cells (9, 10). An essential GATA-4 co-factor, friend of GATA (FOG)-2 is also expressed in murine Sertoli and Leydig cells (11, 12), and it is known that FOG-2 interaction is required for

proper GATA-4 function in the testis. Accordingly, in mice homozygous for a *Gata4* variant that cannot interact with FOG-2 or in mice homozygous for *Fog2* null mutant, Sertoli cell differentiation is blocked resulting in abnormal testis morphogenesis presumably due to an abnormal *SRY* expression (5). No human mutations in these factors leading to abnormal testicular phenotype have been described so far.

The expression of GATA-4 in germ cells has not been satisfactorily demonstrated, except for one study suggesting its presence in fetal gonocytes (9). Testicular germ cell tumors (TGCTs) provide a unique model to study pathways of early germ cell differentiation and somatic transformation. TGCTs that occur in young adults develop from a common precursor, the carcinoma *in situ* (CIS) cell (13). CIS cells are phenotypically

nearly indistinguishable from fetal gonocytes and most likely originate from these cells (14). After puberty, CIS transforms into overt tumors divided into two main histological subtypes, seminomas and non-seminomas. Seminomas retain germ cell features and have a very similar gene expression profile to CIS cells, whereas non-seminomas no longer express germ cell-specific genes and may contain several types of somatic tissues (15).

In this study, we exploited above-described features of TGCTs to study the possible role of GATA-4 transcription factor in early germ cells and during malignant transformation. For this purpose, we analysed GATA-4 expression in testicular CIS and overt TGCT, as well as in human fetal testis. In addition, we investigated GATA-4's essential co-factor FOG-2, the closely related family member GATA-6, and downstream target genes *SF1*, *AMH*/Müllerian-inhibiting substance (MIS), and *INHα* in the same tissue samples.

Tissue samples and methods

Tissue samples

The Regional Committee for Medical Research Ethics in Denmark has approved the use of anonymized human tissue samples for gene expression studies.

The ten normal fetal (gestational weeks 15–41) testis samples were obtained from tissue archives of the Department of Pathology at the Rigshospitalet, Copenhagen, Denmark. Tissues were stored after autopsy of material from induced or spontaneous abortions and stillbirths, mainly due to placental or maternal problems. The fetal age was estimated from the date of the last menstrual bleeding, supported by the foot length of the fetus. Specimens of testicular tumors and the adjacent tissue, which usually contains CIS, were obtained after therapeutic orchidectomy. The fetal tissue samples were fixed in neutral buffered formalin (4%), whereas adult tissues were fixed either in buffered formalin or 4% paraformaldehyde, embedded in paraffin, and sectioned. In total, 10 CIS and 19 tumor samples (six seminomas and 13 non-seminomas) were analyzed using immunohistochemistry.

Immunohistochemistry

The antibodies used for immunohistochemistry were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): AP-2γ (Catalogue no. sc-12762, 1:200), FOG-2 (sc-10755, 1:1000), GATA-4 (sc-1237, dilution 1:200), GATA-6 (sc-9055, 1:500), OCT4 (POU5F1) (C-20, sc-8629, 1:250); from Affinity BioReagents (Golden, CO, USA): SF-1 (PA124565, 1:2000); from Serotec (Oxford, UK): inhibin-α (MCA951s, 1:500); and from Dako (Glostrup, Denmark): α-1-fetoprotein (AFP) (A008, 1:600), human chorionic gonadotropin (A0231, 1:1500). The MAB to AMH (MIS) was a gift from Dr R Cate (Biogen, Cambridge, MA, USA).

The paraffin-embedded tissue sections were deparaffinized and rehydrated using descending concentrations of ethanol. Antigen retrieval was performed by incubating the slides in 0.1 M citric acid (pH 8.0) at ~100 °C for 20 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 5 min. The incubation with primary antibody was carried overnight at 4 °C. An avidin–biotin immunoperoxidase system was used to visualize bound antibody (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA), with 3,3'-diaminobenzidine (Sigma) or aminoethyl carbazole (Zymed, San Francisco, CA, USA) as chromogens. The sections were counterstained with hematoxylin. In negative control experiments, PBS replaced the primary antibody.

The stained sections were analyzed under a light microscope. Scoring of the antigens was based on the staining intensity of at least 10% of the tumor cells, divided into three categories: +, positive staining; +/–, heterogeneous staining; and –, no staining.

Results

GATA-4 is expressed in early fetal gonocytes

The expression of GATA-4 during fetal testis development was examined using a series of samples listed in Table 1. Gonocytes were identified by their morphology and staining for AP-2γ, which in the fetal testis is a gonocytes-specific marker (16). In early fetal testis (15th gestational week), a subpopulation of gonocytes expressed GATA-4 (Fig. 1, Table 1). After the 17th gestational week, GATA-4 was no longer detectable in germ cells. In contrast, GATA-4, FOG-2, SF1, and INHα were expressed in fetal somatic cells (Sertoli and Leydig cells) in almost all fetal samples (weeks 15–41). GATA-6 was expressed only in a subpopulation of early fetal Sertoli cells. AMH was expressed in fetal Sertoli cells of all fetal ages but not in germ cells.

In the adult testis, GATA-4 was positive in Sertoli and Leydig cells, as previously described (9). Normal germ cells were negative for GATA-4, except for a heterogeneous staining of acrosomes in subset of spermatids, which was apparently unspecific. GATA-6, FOG-2, SF1, and INHα were mostly negative in germ cells, whereas Sertoli and Leydig cells expressed GATA-6, FOG-2, SF1, and INHα, as described earlier (10, 12, 17).

Differential expression of GATA-4 and its target genes in CIS cells and overt germ cell tumors

Most of the CIS samples expressed GATA-4 (Fig. 2). GATA-4 expression in CIS cells was heterogeneous, positive cells ranging from 24 to 93% of all CIS cells in a given tubule, and with variable intensity. In CIS cells, the staining was localized into the nucleus and

Table 1 Expression of GATA-4, GATA-6, and co-factor friend of GATA (FOG)-2 and their target genes in fetal germ and Sertoli cells, carcinoma *in situ* testis (CIS), and testicular tumors.

Tissue type	Fetal age (weeks)	GATA-4		FOG-2		GATA-6		AMH/MIS		SF-1		INH α	
		Gc	Sert	Gc	Sert	Gc	Sert	Gc	Sert	Gc	Sert	Gc	Sert
Fetal testis	15	+/-	+	-	+/-	-	+/-	-	+	-	+/-	+	+
	15	+/-	+	-	-	-	+	-	-	-	+/-	+	+
	17	-	+	-	+	-	-	-	+	-	+	-	+
	18	-	+/-	+	+/-	-	-	-	+	-	-	-	+
	18	-	+	-	+	-	+	-	+	-	+	-	+
	20	-	+	-	+	-	+	-	+	-	+	-	+
	20	-	+	+	+	-	+	-	+	-	+	+	+
	24	-	-	-	+	-	-	-	+	-	-	-	-
	39	-	+	-	+	-	-	-	+	-	-	-	+
	41	-	+	+	+	-	-	-	+	-	+	-	+
CIS/adjacent tumour	Age (years)	GATA-4		FOG-2		GATA-6		AMH/MIS		SF-1		INH α	
		CIS	Sert	CIS	Sert	CIS	Sert	CIS	Sert	CIS	Sert	CIS	Sert
CIS/NSE	28	+	+	+	-	+	+	-	+ ^a	+	+	-	+
CIS/NSE+SE	27	+	+	+/-	+	+/-	+	-	-	+	+/-	-	+
CIS/SE	35	+/-	+	+	+	+	+/-	-	+ ^a	+	+/-	+	+
CIS/SE	29	+/-	+	+	+	+/-	+/-	-	+ ^a	+/-	+	+	+
CIS/SE	32	+/-	+	+	+	+/-	+	-	-	+/-	+/-	-	+
CIS/NSE	20	+/-	+	+	+	+/-	+	-	-	-	+	+/-	+
CIS/NSE	32	+/-	+	+	+	+/-	+	-	-	+/-	+/-	-	+
CIS/NSE	28	+/-	+	+	+	-	+	-	-	+/-	+	-	+
CIS/NSE	23	+/-	+	+/-	+	+/-	+	-	+ ^a	+/-	+	-	+
CIS	27	+/-	+	-	+	-	+	-	-	+/-	+	-	+
Overt tumour		GATA-4		FOG-2		GATA-6		AMH/MIS		SF-1		INH α	
SE	22	+/-		+/-		+/-		-		-		-	
SE	29	+/-		+/-		-		-		-		-	
SE	29	+/-		-		+		-		+		-	
SE	33	+/-		-		+/-		-		-		-	
SE	43	+/-		-		+/-		-		-		-	
SE	37	+/-		-		-		-		-		-	
EC	32	-		-		-		-		-		-	
EC	34	-		-		-		-		-		-	
EC	32	-		-		-		-		-		-	
EC	35	-		-		-		-		-		-	
EC	26	-		-		-		-		-		-	
EC	31	-		-		-		-		-		-	
EC	27	-		-		-		-		-		-	
EC	21	-		-		-		-		-		-	
EC	38	-		-		-		-		-		-	
YST	35	+/-		-		+/-		-		+/-		-	
YST	26	+/-		+/-		+/-		-		+/-		-	
YST	27	+		+/-		+		-		+/-		-	
YST	30	+		-		+		-		+/-		-	
YST	21	+		-		+		-		+/-		-	
CHC	26	-		-		-		-		-		-	
CHC	31	-		-		-		-		-		-	
CHC	36	-		-		-		-		-		-	
TE	35	+/-		+/-		-		-		-		-	
TE	27	+/-		+/-		-		-		-		-	
TE	38	-		-		+/-		-		+/-		-	

Gc, germ cell; Sert, Sertoli cell; CIS, testicular carcinoma *in situ*; AMH/MIS, Anti-Müllerian hormone/Müllerian-inhibiting substance; SF-1, steroidogenic factor 1; INH- α , inhibin- α ; SE, seminoma; NSE, non-seminoma; EC, embryonal carcinoma; YST, yolk sac tumor; CHC, choriocarcinoma; TE, teratoma. NSE tumors were composed of EC, YST, CHC, and TE components.

^aAMH positivity was observed only in visibly undifferentiated Sertoli cells in a few tubules.

Figure 1 Immunohistochemical expression of AP-2 γ (A and C) and GATA-4 (B and D–F) in fetal testes on gestational week 15 (A–D), 17 (E), and 20 (F). Original magnification $\times 20$ (A and B), $\times 40$ (C–F). Scale bar 200 μ m. Gonocytes are marked with arrows.

cytoplasm in contrast to the strong nuclear-only staining in Sertoli cells (Fig. 2B). FOG-2 expression was detected in most of the CIS cells, its expression being uniform in most of the samples. GATA-6 was expressed heterogeneously in most of the CIS cells. As to GATA-4 target genes, SF1 was heterogeneously expressed in most of the CIS cells, but INH α was mostly absent, and AMH totally negative in CIS cells of all samples.

Most of the seminomas expressed heterogeneously GATA-4 and GATA-6 (Fig. 3A and D, Table 1). Staining for FOG-2, AMH, SF1, and INH α was mostly negative. Non-seminoma tumors were mostly composed of different histological components, and yolk sac, choriocarcinoma, and embryonal carcinoma components within these tumors were identified by staining for AFP, human chorionic gonadotropin, and Oct-3/4 respectively. Of the non-seminomas, embryonal carcinomas and choriocarcinomas did not express GATA-4, FOG-2, GATA-6, SF-1, or INH- α (Fig. 3B, E and H, Table 1). In contrast, yolk sac tumors abundantly expressed GATA-4, GATA-6, and target gene SF-1, whereas they remained negative for the other studied GATA target genes AMH and INH α (Fig. 3C, F and I, Table 1). In addition, immature teratomas expressed GATA-4 and FOG-2, but not the other genes studied.

Discussion

In this study, we unequivocally demonstrated the expression of GATA-4 in a subpopulation of early human fetal germ cells. This finding is in line with our

previous study suggesting GATA-4 expression in early fetal gonocytes from the 12th week of gestation (9). We confirmed in the current study that the GATA-4 positive cells are gonocytes by examining in parallel the expression of a gonocytes-specific marker, AP-2 γ in serial sections. Like GATA-4 (this study), several proteins are downregulated in germ cells during the early fetal development, while early gonocytes differentiate to spermatogonia (18, 19). These proteins include pluripotency markers OCT-3/4 and NANOG, and AP-2 γ , the germ cell expression of which is declining gradually from the second trimester of gestation onwards (16, 20). GATA-4 thus adds to the list of these early human gonocyte markers.

CIS cells are considered to be arrested and transformed gonocytes, but the details of the molecular pathophysiology of CIS are not well understood (14, 19). Surprisingly, in this study, we found that CIS cells expressed GATA-4. The possible role of GATA-4 in CIS remains unraveled, but it is noteworthy that enhanced GATA-4 expression has also been reported in other tumor types such as adrenal cortical carcinomas, and it has been speculated to serve as a survival factor for tumor cells (21). Interestingly, GATA-4 expression within a given sample was not uniform but rather heterogeneous, without any association with the histological type of the adjacent tumor. The significance of this finding remains unclear, but heterogeneous expression in CIS cells has also been reported for some other proteins, such as TRA-1-60, a marker of pluripotency (16). As to TRA-1-60, the heterogeneous

Figure 2 Immunohistochemical expression of GATA-4 (A and B), its coregulator FOG-2 (C), GATA-6 (D), and target genes SF-1 (E) and INH α (F) in testes with CIS. Original magnification $\times 10$ (A), $\times 40$ (B–F). Scale bar 200 μ m. CIS cells are marked with arrows.

Figure 3 Immunohistochemical expression of GATA-4 (A–C), GATA-6 (D–F), SF-1 (G–I) in seminoma (A, D and G), embryonal carcinoma (B, E and H), and yolk sac tumor (C, F and I); embryonal carcinoma component is identified by its expression of Oct-3/4 (inset in B), and yolk sac tumor component is identified by its expression of AFP (inset in C). Original magnification $\times 40$. Scale bar 200 μm .

pattern is presumed to be linked to the progressive genomic aberrations or reprogramming to lead to invasive tumors (22). In contrast to GATA-4 and TRA-1-60, pluripotency markers OCT3/4 and AP-2 γ are homogeneously expressed in CIS cells (16, 23).

GATA-4 is essential for testicular development and executes its functions together with its co-factor FOG-2 (5). In *Fog2*^{-/-} mice, *Sry* expression is reduced, and *Sry* downstream genes *Sox9* and *Amh* are downregulated. In our study, we investigated the expression of GATA-4 and its cofactor FOG-2 as well as a set of essential testicular target genes in CIS samples. Our finding of the simultaneous expression of the essential GATA-4 cofactor FOG-2 in most of the CIS cells suggests that GATA-4 can function to drive target gene expression in CIS cells. However, only one known GATA-4 target gene, *SF-1*, was also expressed in CIS cells, whereas other testicular GATA-4 targets, *INH α* and *AMH* were not present. Although it is not possible to draw definitive conclusions on the biological significance of these findings based on immunohistochemistry, the differential expression of the presumed GATA-4 target genes suggests distinct role of this transcription factor in CIS cells as compared to normal gonadal somatic cells.

We speculate that the expression of GATA-4 in a subset of CIS and seminoma cells reflects their gonocyte-like phenotype. Gonocytes retain some features of embryonic stem cells, most notably a high expression of pluripotency-related genes, such as OCT3/4 and NANOG (14, 18, 19). In this study, we demonstrated also the presence of GATA-4, which is a marker for early somatic differentiation of the inner cell mass (ICM) (24), in early gonocytes. We hypothesize that GATA-4 may be one of the genes upregulated during the germ cell specification from ICM. The absence of GATA-4 in

embryonal carcinoma, which is a malignant equivalent of pluripotent embryonic stem cells that lost germ cell lineage characteristics, is in line with this hypothesis.

In the ovary, GATA-4 is expressed in a subpopulation of dysgerminomas (75%) (25), which is a tumor with a very close gene expression profile as compared to testicular seminomas (26). Here, we find that also most of the testicular seminomas are positive for GATA-4. Similarly to dysgerminomas (25), seminomas were mostly negative for the GATA-4 cofactor FOG-2, suggesting that GATA-4 may not function properly in seminoma tissue due to a lack of the presence of this essential cofactor. Although GATA-6 has not been detected in ovarian dysgerminomas (25), we now show that most of the testicular seminomas express this transcription factor. Taken together, although certain similarities between the corresponding ovarian and testicular tumors exist, there are also clear differences in their gene expression patterns. Thus, the role of GATA factors and their cofactors may differ in germ cell tumors in the male and female.

Within the non-seminoma group of testicular tumors, yolk sac tumor components abundantly expressed GATA-4 as well as GATA-6 in contrast to their absence in embryonal carcinomas and choriocarcinomas. In line with this study, GATA-4 and GATA-6 expression has also been demonstrated in pediatric testicular as well as non-gonadal yolk sac tumors (27, 28). Moreover, GATA-4 expression has been reported in adult ovarian and testicular yolk sac tumors (25, 29).

Interestingly, GATA-4 appears to regulate different pathways in Sertoli cells before and after the onset of spermatogenesis. AMH, one of the presumed targets of GATA-4, is only expressed in immature Sertoli cells,

whereas the expression of GATA-4 remains activated in adult testes. In adult testes harboring CIS cells, AMH was only expressed in some visibly undifferentiated Sertoli cells, which is a sign of testicular dysgenesis (30–32).

We herein demonstrate the expression of GATA-4 in a subpopulation of early fetal germ cells, presumably gonocytes, and its downregulation during differentiation of germ cells to spermatogonia. Reflecting this developmental pattern, GATA-4 is heterogeneously positive in CIS cells and seminomas, the germ cell neoplasms of adult testes that retain the immature gonocyte-like phenotype. In contrast, GATA-4 was not detectable in non-seminomas with embryonic features.

In conclusion, we find that GATA-4, GATA-6, and FOG-2 proteins are present in CIS cells, which concomitantly also express the GATA downstream target gene *SF-1*. The constellation of the GATA target genes between various testicular cell types does differ, as indicated by the abundance of AMH and INH α in Sertoli cells and their absence in CIS cells. The present findings also support the early fetal origin of CIS cells, and suggest that GATA-4 is involved in early germ cell differentiation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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