Original article:

Intratubular germ cell neoplasia of the testis identification by argyrophilic nucleolar organiser region (AGNOR) quantification

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Abstract:
Silver nucleolar organizer regions (AgNOR) were studied in 88 Orchidectomy specimens and testicular biopsies and were subjected for AgNOR staining. These included five orchidectomy specimen as control group where testes were removed for nonneoplastic conditions, 11 cases of germ cell tumors, 62 testicular biopsies for infertility, 9 undescended testes and a case of somatosexual ambiguity. The mean AgNOR counts showed a progressive and statistically significant increase from normal testicular tissue through Intratubular germ cell neoplasia (ITGCN) to testicular germ cell neoplasms. The AgNOR technique is simple, inexpensive and a useful adjunct to histopathology to identify ITGCN, which have a higher risk for development of invasive germ cell neoplasms. The count, however, cannot be standidized for a particular lesion as there are inter laboratory variations.

Key words: AgNOR, ITGCN, Germ cell testicular neoplasms

Introduction
Nucleolar organizer regions (NORs) are chromosomal segments in which ribosomal RNA (rRNA) in encoded. In the human karyotype, NORs are located on each of the short arms of the five acrocentric chromosomes i.e 13, 14, 15, 21 & 22. A silver staining technique is used to identify the acidic non-histone proteins associated with NORs, called AgNORs, and they have evoked much interest because of the claims that their frequency within nuclei is significantly higher in malignant cells than in normal, reactive or benign neoplastic cells.\(^{(1)}\) Germ cell atypia is an interesting observation made in testicular biopsies of patients who were considered to have a high risk of developing testicular neoplasm. Atypical cells are large gonial cells with clear cytoplasm and nuclei about twice the size of the normal with irregular clumped chromatin.\(^{(2)}\) This change has been adequately demonstrated to be a true precursor of invasive germ cell tumors.\(^{(3)}\) The atypia has been found inside the semineferous tubules most commonly and the entity is named as intratubular germ cell neoplasia (ITGCN),\(^{(4)}\) carcinoma in situ \(^{(5)}\) and stage ‘o’ carcinoma.\(^{(5)}\) Infertility is not an uncommon feature in patients with testicular tumors. The occurrence of ITGCN in infertility has been reported by a few.\(^{(6)}\) The identification of ITGCN is possible by study of H & E stained sections for presence of morphologically large abnormal germ cells and by Agnor counts in atypical cells. Using above techniques, ITGCN can be detected in seminiferous tubules adjacent to germ cell tumors, testicular
biopsies in infertility cases and in cases of cryptorchidism.

**Material and methods**

The present study of 88 cases consisted of

1. Orchidectomy specimen without neoplasia
2. Orchidectomy specimen with germ cell neoplasia
3. Testicular biopsies from infertility cases
4. Resected undescended testis with or without neoplasia
5. Somatosexual ambiguity

After noting clinical history and gross features the specimens and biopsies were fixed in 10% formalin and then subjected to routine histological processing. Four micron thick sections were taken from paraffin blocks and stained by H & E and AgNOR.

**Staining procedure for NORs**: This was carried out as per the method of Crocker et al. (7) the fixed paraffin sections were dewaxed and dehydrated through descending grades of alcohol to distilled water. These sections were incubated in a silver solution for 40 minutes in the dark. The silver colloidal solution was prepared by mixing one volume of 2% gelatin in 1% formic acid solution to two volume of 50% aqueous AbNO₃ solution. The stained slides were washed for 3 minutes in three changes of distilled water. Counter staining was done with 0.01% safranin solution. Sections were dehydrated, cleared and mounted in DPX.

**Enumeration of AgNORs**: It was done according to the techniques described by Orell et al. (8). The sections were studied under oil immersion and AgNORs stained as black dots and were counted within the nuclei. For each slide 50 germ cell nuclei adjacent to the basement membrane were counted. Mathematical and statistical tests were applied to calculate mean AgNOR counts.

**Observations**

Out of the total 88 cases, five specimen from 45-70 year age group who underwent orchidectomy for non-neoplastic causes were studied as control group for AgNOR count in normal spermatogonia. Three cases showed normal testicular tissue histologically (Fig. 1) and two cases showed regional fibrosis and hyalinization. No abnormal germ cells were detected. The AgNOR count ranged from 5.28 to 7.24/nucleus (Fig. 2).

In 8 cases of normally descended testis with germ cell neoplasia in the age group of 22 to 65 years, 2 each were embryonal carcinoma, teratocarcinoma, seminoma and mixed germ cell tumors. In 5 cases adjacent tubules were atrophied and lined by a few layers of atypical cells.

AgNOR counts in intratubular component ranged from 8.6 to 11.26/nucleus while in invasive part it ranged from 9.76 to 14.10/nucleus. 62 cases of infertility and subfertility were grouped into 6 groups according to the abnormalities observed in the seminiferous tubules. The AgNOR counts in the first 5 groups were similar to control group.

All the three cases from the sixth group with histology of tubules lined by abnormal germ cells (Fig. 3) showed high AgNOR counts ranging from 8.5 to 10.10/nucleus (Fig. 4).

Among the 12 undescended testes 7 showed only epididymal tissue with complete atrophy of testicular tissue and hence were omitted from the study. In the remaining 5 cases 3 were replaced by germ cell tumors. Histologically all were seminoma (Fig. 5) with AgNOR count ranging from 8.94 to 11.84/nucleus (Fig. 6). In two cases adjacent tubules showed ITGCN with high AgNOR counts ranging from 9.64 to 11.68/nucleus.

A case of somatosexual ambiguity was noted. The
patient was 8 year old and sex chromatin negative. Histological examination showed tubules lined by sertoli cells and a few large atypical cells. AgNOR count was high compared to the control group being 7.9/nucleus. (Table 1)

Discussion

In humans AgNORs are closely associated with interphase nucleolus. They have been studied extensively, following development of silver staining techniques.(4,9) Recently there have been more reports on the study of AgNORs in malignant cells. DeLozier – Blanchet studied AgNORs in chromosome preparation of human testicular tumors and observed ectopic NORs in 4 of 7 malignant tumors.(10) This qualitative change was thought to represent possible chromosomal rearrangement or reexpression of inactive NORs.

In the present study, AgNOR counts were carried out in 5 normal adult testes and 11 germ cell tumors. AgNOR count for spermatogonia in control group varied from 5.28 to 7.25/nucleus and was higher 8.70 to 13.88 in tumor group. Barbara Loftus et al examined 12 normal testes and 46 testicular tumors and AgNOR counts varied from 2.8 to 5 in control group and from 7.0 to 20 in germ cell tumors.

Abnormal cells in seminiferous tubules adjacent to germ cell tumors have been reported and are considered to be premalignant, this condition has been termed as ITGCN.(10,11,12,13,14)

The criteria for the diagnosis of ITGCN were putforth by Skakkebeak(3) and Sigg & Hedinger(12) are

1) Atrophic tubules with thickened basement membrane
2) Single layer of spermatogonia with large nuclei and nucleoli with abundant clear cytoplasm
3) Sertoli cells pushed towards the centre of the tubules

Klein et al studied 111 orchidectomy specimens from patients with germ cell tumors for the presence of ITGCN in adjacent testicular tissue. Of these specimens, 36 out of 44 cases of pure seminoma (82 %) and 41 out of 55 non-seminomatous germ cell tumors (75%) had ITGCN.(15)

Barbara Loftus et al studied 43 cases of testicular germ cell tumors and diagnosed ITGCN in 30 cases (70 %) by H & E study and in 36 cases (84 %) by AgNOR count which varied from 7.9 to 15.0/nucleus.(16)

In the present study out of 8 cases of germ cell tumors, adjacent testicular tissue could be identified in only 5 cases. In all of them the testicular tubules appear atrophied and lined by single layer of atypical germ cells. The AgNOR count was high (Table 1).

The increased of developing germ cell tumor in undescended testis has been well established. The chances of malignancy increases by 30 to 50 times than in a normally descended testis.(11)

In the present study the incidence of germ cell tumor in undescended testis was found in 3 out of 12 cases (25 %). According to mostofi et al seminoma is is a frequent germ cell tumor in cryptorchids. In the present study also all the 3 cases showed features of seminoma. Among these 3, ITGCN was observed in 2 cases. The AgNOR count was high in the intratubular component of the neoplasms.

In many studies of testicular biopsies from infertile, oligospermic men, the incidence of ITGCN varied from 0.39 to 1.10 % (sigg & Hedinger, skakkebaek, pryor). In the present study 62 cases of infertility were studied for evidence of ITGCN. Abnormal germ cells were detected in 3 cases (4.84 %) only. The
AgNOR count was high ranging from 8.50 to 10.10/nucleus.

Conclusion
From the present study following conclusions were drawn
1) Demonstration of ITGCN in tubules adjacent to different types of germ cell neoplasms indicate their common origin - the germ cell.
2) In infertile males, atrophied testis is one of the predisposing causes of germ cell tumors.
3) Undescended testis also have predilection for development of germ cell neoplasms.
4) AgNOR study is helpful in identifying abnormal germ cells in ITGCN and follow up of these cases may be helpful in early detection of germ cell neoplasms.

References
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