Original article

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THE KI-67 CELL PROLIFERATION MARKER IN HUMAN METANEPHROGENESIS

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The kidney plays several essential roles, including the excretion of metabolic wastes, maintenance of key homeostatic parameters of the blood plasma, participation in blood pressure and hormone levels regulation. These diverse functions are enabled by the developmental process that provides the presence of specific cells for performing all diverse functions. Organogenesis of the kidney is an intricate mechanism involving cell proliferation as a fundamentally necessary process. The aim of this study was to determine proliferative activity during the metanephros stage of renal development, based on the spatial and temporal expression pattern of the cell proliferation marker Ki-67. Kidney tissue specimens of 30 human fetuses with gestational ages ranging from 11 to 36 weeks were analyzed. The specimens were divided into three groups based on gestational age, each corresponding to the earlier, mid or late gestation period. Routine histological processing yielded tissue sections. The proliferative activity of the cells (expression of the Ki-67 protein) was examined by an immunohistochemical assessment of Ki-67, according to the manufacturer's protocol. The presence of Ki-67-positive cells characterized all metanephric structures but with different intensity. The most prominent expression was revealed in the nephrogenic zone in the earlier weeks of development, indicating the role of cell proliferation in nephron formation. The intensity of Ki-67 antigen expression gradually decreased in all cortical structures until the end of the trial period. In the metanephric medulla, the proliferation was less pronounced only after week 20, and the only Ki-67 positive cells were single cells of collecting duct epithelia, narrow parts of Henle's loops and the interstitium. Cell proliferation was continuously present during metanephrogenesis. It was characterized by different intensity, more pronounced in the nephrogenic zone and renal cortex due to the dominant presence of cells in their structural components. However, the obvious developmental remodeling of the kidney tissues inevitably indicates the need to correlate proliferation with other developmental processes, apoptosis above all.

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Introduction

The kidneys are the central organs of the urinary system which perform numerous functions important for normal postnatal life. The basic functions of the kidneys are the excretion and removal of harmful products of organic decomposition of molecules from the blood plasma. The kidneys excrete approximately 200 liters of blood per day by filtration. Equally significant is the homeostatic regulation of blood plasma parameters, which includes the regulation of its ionic concentration (the balance between water and salt, acidic and basic molecules) and preserves useful molecules, which also regulates plasma volume and pressure and stabilizes blood pH. These renal functions are inextricably related to their endocrine function. By secreting renin, the kidneys become part of the regulatory RAAS system (Renin-Angiotensin-Aldosterone System), a hormonal system that by secreting signaling molecules regulates blood pressure as well as systemic vascular resistance (1).

These different functions are enabled by the phylogenetic and ontogenetic development of the kidneys, which results in the presence of cells as a tool for performing all complex functions. This process is conventionally called “nephrogenesis”, but even though millions of nephrons are the basis of its function, they are only one part of its

structure. A large part of the parenchyma is occupied by a branching system of collecting channels, a function of the organ-specific connective tissue of the interstitium and cortex, and especially the medulla as well as a single vascular bed (1).

The formation of all organs during the embryonic and fetal phases of intrauterine development, including the kidneys, depends on the spatially and temporally dependent expression of numerous signaling polypeptide factors that regulate cell mitotic activity and coordinated cell death (2-4). In the same way, nephrogenesis includes a highly controlled series of morphogenetic events within the area of the determined cell mass of the intermediate mesoderm on the dorsal side of the embryo (5). Starting from the fourth week of development, three pairs of developmental "kidneys" form consecutively in the embryo: pronephros, mesonephros and metanephros. Although they do not last for a long time and they degenerate in the earliest phases of organogenesis of the urinary systems, the pronephros and mesonephros are developmental stages that have a significant inductive effect on the formation of metanephros from which the final kidney structures will develop (6, 7).

The metanephros, a precursor of the final kidney, is built at the very beginning of development from two basic types of defined cells: epithelial cells of the ureteric bud whose origin is the urethra of the mesonephros and mesenchyme in its environment, whose cells condense to form a metanephric blastema.

Both cell types, ureteral (ductogenic) and mesenchymal (nephrogenic) are subjected to a repeated series of inductive signals that serve to organize the complex architecture of the renal parenchyma (8-10). A series of mutually inductive interactions between these tissues causes the urethral bud to sequentially branch and form the ureter, renal pelvis, calyces, and collecting tubules, while the mesenchyme undergoes a complex process of mesenchymal transition into highly specialized populations of different types of nephron epithelial cells from the renal corpuscle to the end of the distal tubules. The third type of cells, interstitial cells, also differ from the metanephrogenic mesenchyme (4, 11, 12).

Renal organogenesis is based on a balanced course of events on a cellular level, such as proliferation, programmed death—apoptosis, differentiation, and morphogenesis (13-15). Despite the fact that the research is now being conducted on the level of molecular biology, these events have not yet been definitively explained. In several peer-reviewed studies (2, 3, 11-14, 16), the authors presented their most recent findings on the complex dynamics of renal development (spatially and temporally coordinated gene expression activity and the consequent presence of numerous protein factors that regulate mitotic cell activity and programmed cell death) but also warned of the fact that the results were obtained from research on experimental animals because such research is not feasible on human material due to ethical limitations (15-17).

The kidney of an adult develops from less than a thousand cells at the beginning of the development process up to several million at the end of the process, and it is self-evident that organogenesis requires extensive and accelerated cell proliferation. After induction, proliferation is an event that begins in the most vulnerable period of intrauterine development; it continues for weeks under different influences and thus carries the greatest risks for the occurrence of anomalies (18-20). Impaired proliferation and cell death have been shown to be associated with renal abnormalities involving agenesis, dysplasia, hypoplasia, obstructive uropathy, and vesicoureteral reflux, which can lead to chronic renal failure in children (21).

Understandably, proliferation as a subject of interest is still a very current topic. Standard research and diagnostics use typical representatives, which include immuno-histochemical protein markers of proliferation and apoptosis: Ki-67 and representatives of the Bcl-2 family of proteins.

Ki-67 is a non-histone nuclear protein whose presence is differently expressed during the phases of the cell cycle. In the interphase, it can be detected only inside the nucleus, while in mitosis the protein is located on the surface of condensed chromosomes. The fact that the Ki-67 protein is present in all active phases of the cell cycle (G1, S, G2, and mitosis) and that it is absent in quiet (early G1 and G0) cycles, as well as that its presence increases during cell preparation for division, allows immunohistochemical techniques to use Ki-67 as an excellent marker of cell proliferation; the more immunopositive cells, the more cell divisions. Thus, over time, this protein has become a proliferative marker, an important and reliable indicator and indirect measure of the growth fraction of the examined cell population, as well as a prognostic marker in the diagnosis of various phenomena and conditions (22).

Aim

This study aimed to examine the morphological aspects of the metanephric development in kidney samples of human fetuses of different gestational ages, and based on the presence and distribution of immunopositive cells of Ki-67 proliferation markers to determine the spatially and temporally different proliferative activity of cell-building types during the early and late weeks of the metanephros stage of kidney development.

Material and Methods

Material

The material consisted of the kidneys of 30 human fetuses of both sexes, aged 11 to 36 weeks of development, who died suddenly in utero or were autopsied within 24 hours after birth. The material was obtained from the Clinic of Pathology, Clinical Center of Niš. Fetal tissues were treated as autopsy material with the permission of the Ethics Committee of the Faculty of Medicine in Niš (No. 12-6329/4).

The fetuses were examined macroscopically; their weight and crown-rump length were measured; their gestational age was expressed in weeks of intrauterine development.

The study included only those fetuses that did not show any signs of maceration. The material was divided into three groups where Group 1 included fetal kidneys of the gestational age of 11 to 15 weeks, Group 2 of the gestational age of 15 to 28 weeks, and Group 3 of the gestational age of 28 to 36 weeks.

Methods

The kidney samples were fixed in 10% buffered formalin. Routine histological processing provided paraffin blocks that were cut into 5-micrometer thick tissue sections.

For the purposes of histological analysis and assessment of the morphological properties of metanephros of different ages, tissue sections were stained by the standard hematotoxylin-eosin (HE) method.

Proliferative cell activity (detection of the Ki-67 protein) by monoclonal antibody against Ki67 was performed with the use of a monoclonal antibody Ki-67, Clone MIB-1, (Code M7240, Dako, Denmark; dilution 1:75), according to the manufacturer's protocol (23).

Results

In the first group (the gestational age 11 to 15 weeks), the Ki-67 antigen was expressed by cells of the nephrogenic zone, where the reaction was the most intense in the structures of the most superficial part of the cortex, and the immunopositivity of the cells of the inner cortex and medulla was lower (Figure 1a). The strongest Ki-67 immunopositivity was observed in the outer part of the cortex, in the cells of the developmental forms of future nephrons (vesicles and S-forms). There were individual Ki-67 positive cells in the branches of the urethral bud, ampoules and tubular parts (Figure 1b). In the inner part of the cortex, in the formed renal corpuscles, individual cells of Bowman's capsule (the parietal and visceral leaf) were Ki-67 immunopositive (Figure 1b). In the interstitium, a smaller number of mesenchymal cells between the tubular structures showed a positive reaction to the Ki-67 antigen. The cells of the epithelium of the renal pelvis and renal calyces did not express the Ki-67 antigen (Figure 1a).

In the second group (the gestational age 15 to 28 weeks), up to 21 weeks of kidney development the Ki-67 immunopositive reaction remained in the very superficial part of the nephrogenic zone and it was the most intense form just below the capsule (Figure 2).

a) b)

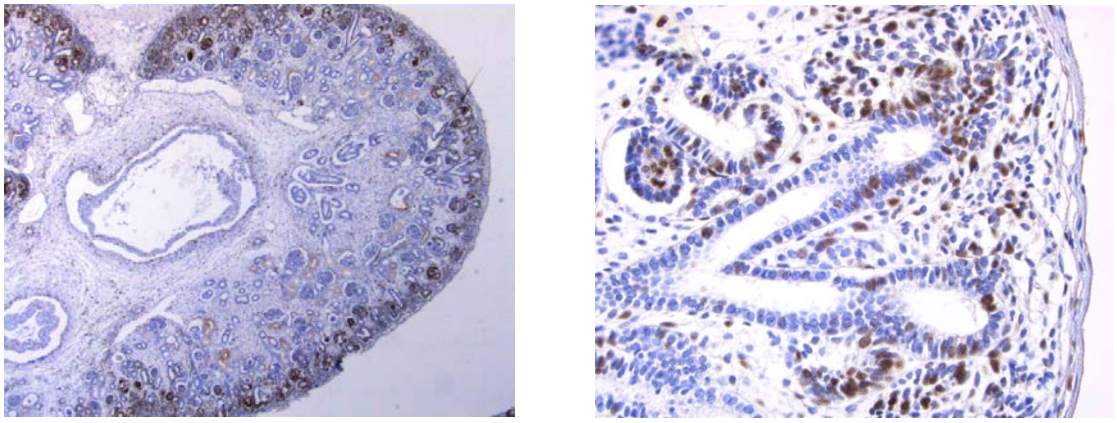


Figure 1. Ki-67 antigen expression in metanephric tissue at 13 weeks of development: a) The strongest expression of the Ki-67 antigen is observed in the nephrogenic zone, completely on the surface of the cortex; epithelial cells of the pelvic urothelium (arrow) do not express the Ki-67 antigen (x40); b) Detail from the previous image: most metanephric blastema cells and vesicles are Ki-67 immunopositive, and only rare single cells in ampoules (asterisks) of the ureteral bud express the Ki-67 antigen (x400)

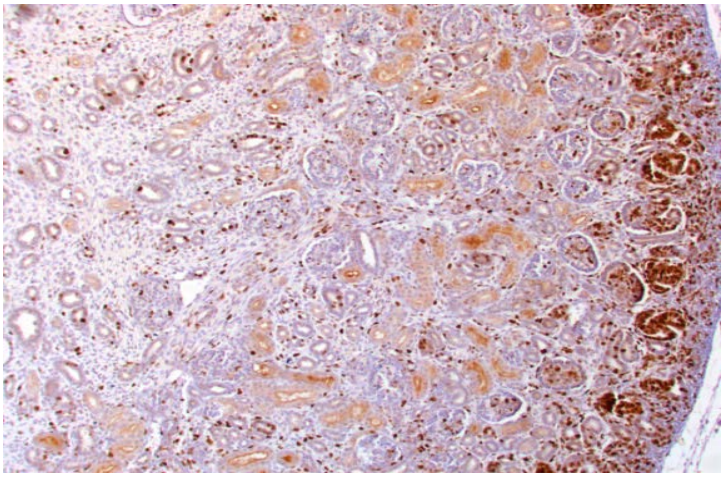


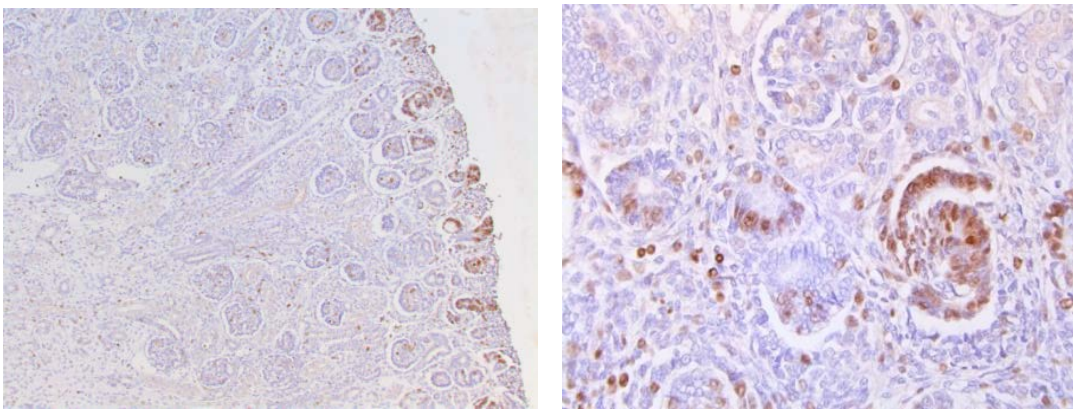
Figure 2. Ki-67 antigen expression in metanephric tissue at 15 weeks of development. The strongest expression of the Ki-67 antigen is observed in the surface layer of the nephrogenic zone. Deeper in the cortex, the immunopositive reaction is seen as a continuous line in the apical region of the epithelial cells of proximal nephron tubules (arrows) and in individual cells within renal corpuscles (x100)

From weeks 21 to 28 of development, the intensity of Ki-67 immunopositivity in metanephric tissue decreased. Only a small number of mesenchymal cells on the surface of the nephrogenic zone showed a weaker expression of the Ki-67 antigen. Somewhat more immunopositive cells were located within the immature renal corpuscles (Figures 3a and 3b). A weak positive reaction was observed in some cells of the proximal tubules (asterisks), while other tubular structures of the Ki-67 were immunonegative (Figure 3b). In the formed renal corpuscles of the internal cortex, the Ki-67 antigen was expressed by individual glomerular cells and their localization coincided with the position of endothelial cells in the capillaries of the glomeruli (Figure 3c). Individual immunopositive cells of collecting ducts and Henle's loops were present in the medulla, as well as rare mesenchymal cells of the interstitium, which sporadically expressed the Ki-67 antigen (Figure 3d).

In about 28 weeks, the strongest Ki-67 immunopositive reaction developed in the thin peripheral layer of the nephrogenic zone (Figures 4a and 4b), and in the medulla, individual cells of the collecting ducts and narrow parts of the Henle's loop (Figure 4c) showed immunopositivity.

In the third group (the gestational age 28 to 36 weeks, Figure 5), the ampoules and the nephrogenic zone disappeared between weeks 32 and 36 of metanephros development, so that the part of the renal cortex that showed the strongest expression of the Ki-67 antigen was not present. Immunopositivity is observed only in individual cells in all kidney structures.

a) b)



c) d)

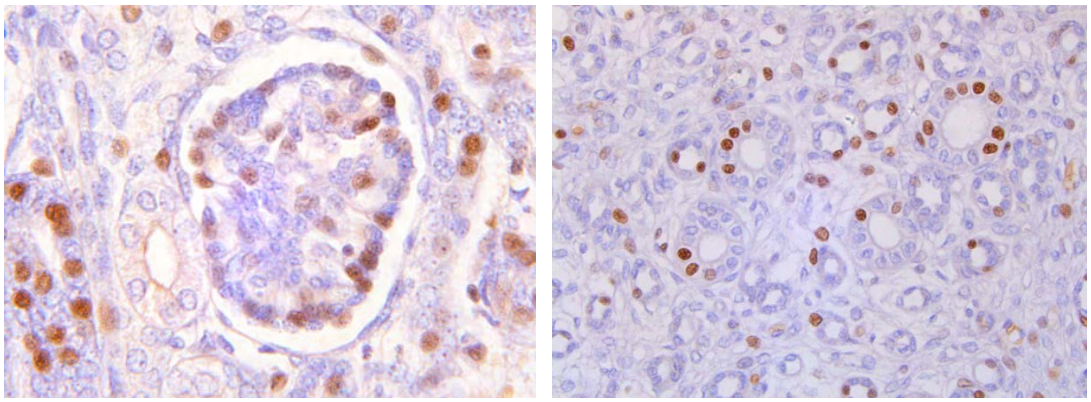
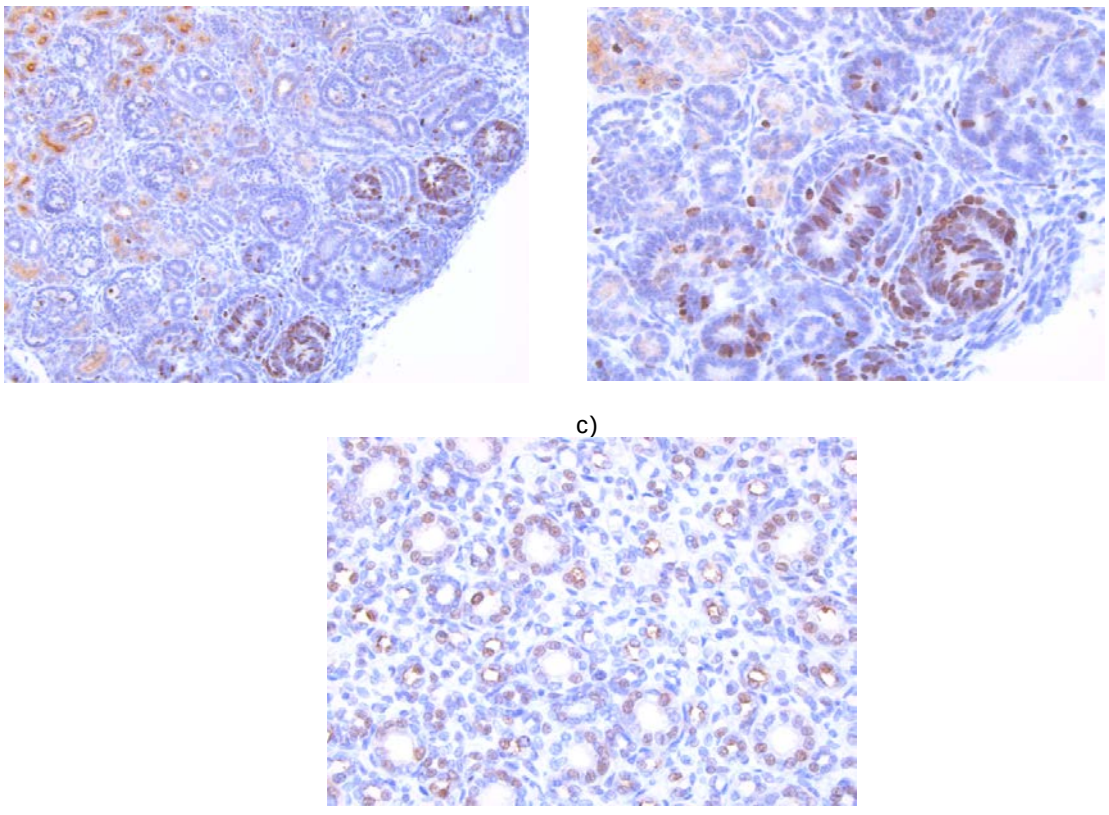


Figure 3. Ki-67 antigen expression in metanephric tissue between weeks 22 and 23 of development. a) A strong immunopositive reaction is observed in the most superficial part of the nephrogenic zone and a weaker immunopositivity is present in the cortex and medulla (x100); b) Part of the nephrogenic zone shows the expression of the Ki-67 antigen in cells of S-shaped the nephron and the forming renal corpuscle; all cells of the future visceral leaf of the Bowman's capsule are highly immunopositive while a smaller number of immunopositive cells are found in the parietal leaf of the Bowman's capsule (x400); c) The formed renal corpuscle contains Ki-67 immunopositive cells of the visceral leaf, while the cells in the parietal leaf of the Bowman's capsule are Ki-67 —immunonegative. x800); d) In the cross-section of the medulla, individual epithelial cells of collecting ducts strongly express the Ki-67 antigen, and in the epithelial cells of the narrow and wider parts of Henle's loop, a sporadic and weaker immunopositive reaction is observed. (x400)

a) b)



c)

Figure 4. Ki-67 antigen expression in metanephric tissue at 28 weeks of development: a) and b) The immunopositive reaction is strongest in immature forms of the nephron in the reduced nephrogenic zone a) x200; b) x400; c) In the cross-section of the medulla, the immunopositive cells belong to the collecting ducts and the narrow parts of Henle's loop (x400)

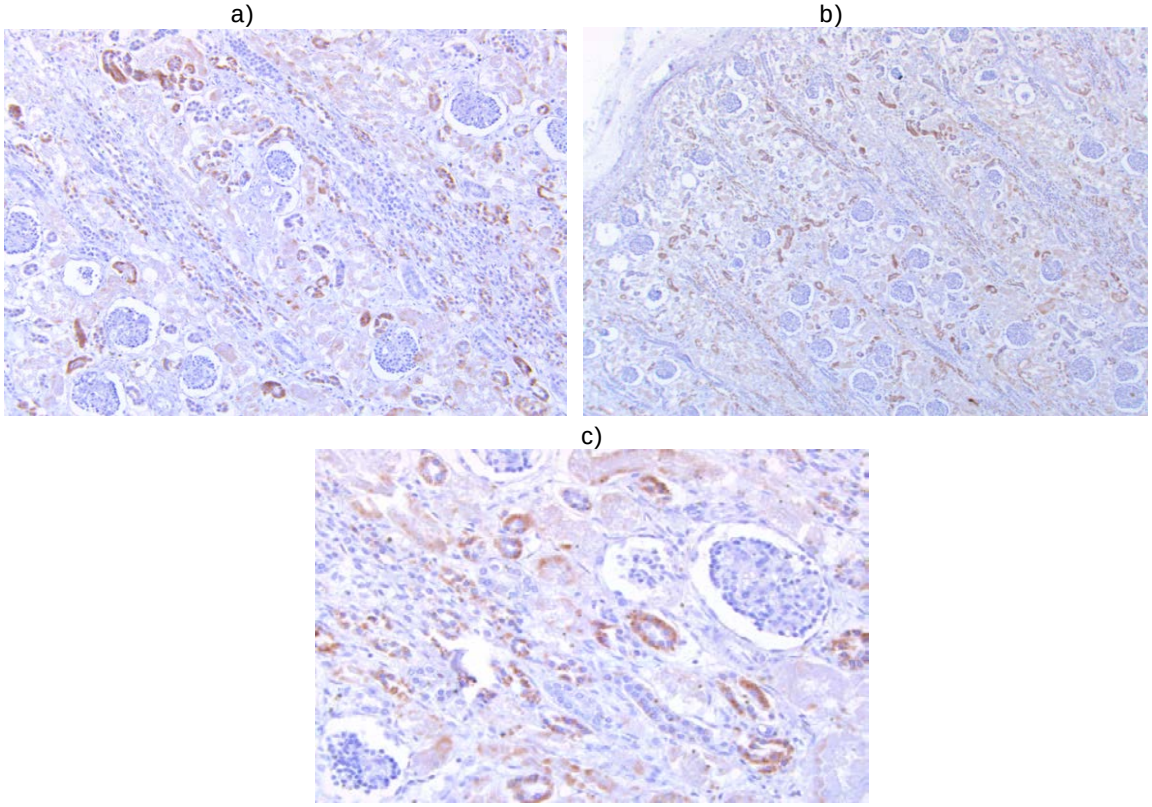
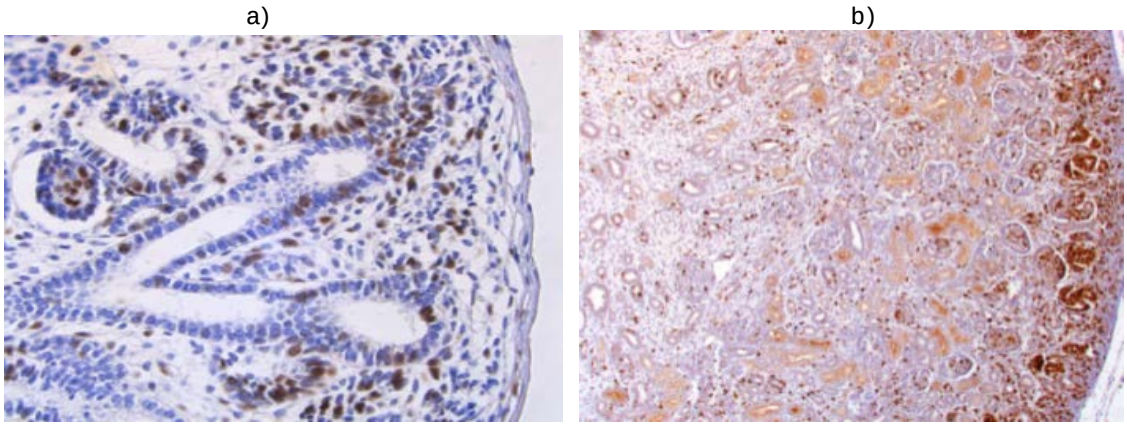


Figure 5. Ki-67 antigen expression in metanephric tissue at 36 weeks of development. A weak fine-granulated cytoplasmic Ki-67 immunopositive reaction is observed in the epithelial cells of the distal tubules of the nephron and the collecting ducts that form Ferrein's pyramids; there is no expression of the Ki-67 antigen in the renal corpuscles, a) x100; b) x200; c) x400

Taking into account the expression of the Ki-67 antigen (Figure 6), it was observed that in the nephrogenic zone, at the beginning of metanephros development (12 to 15 weeks), a strong expression of the Ki-67 (Figures 6a and 6b) was shown by metanephric blastema cells and immature forms of renal corpuscles (vesicles and S-forms). From weeks 19 to 22 of development, the intensity of Ki-67 antigen expression decreased in the renal corpuscles (Figure 6c).

The development of the metanephric medulla began later than that of the cortex, and after week 20, there was a characteristic expression of the nuclear protein Ki-67 in the few epithelial cells of the collecting channels and narrow parts of Henle's loop (Figure 6d); in the same period, the epithelial cells of the collecting ducts were immunonegative. In week 36 of development, the metanephric cortex was formed with all the histological components (renal corpuscles, proximal and distal nephron channels, medullary rays) in which only individual cells of the renal tubular system expressed Ki-67 (Figure 6e).



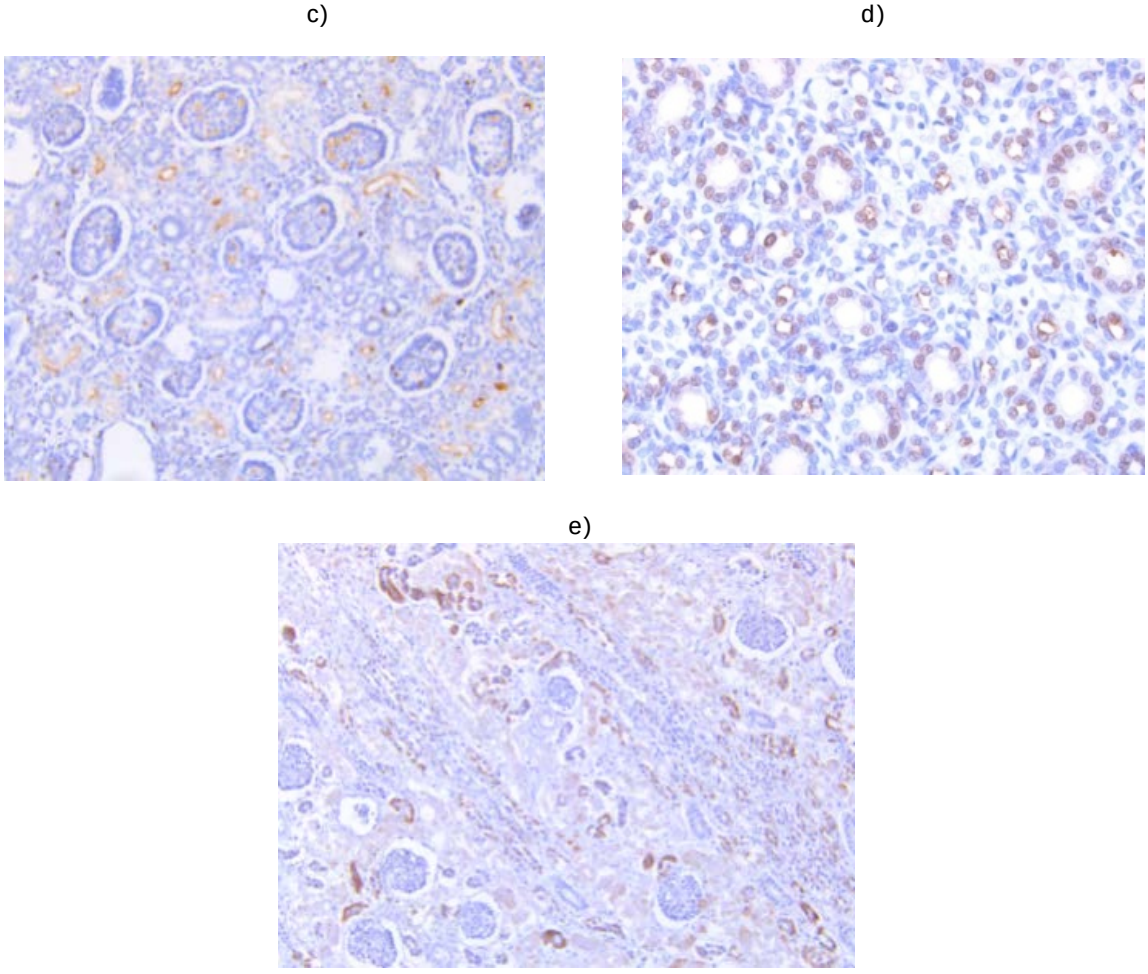


Figure 6. Ki-67 antigen expression in the metanephros in weeks 12–13 (a), 14–15 (b), 19–22 (c), 28 (d) and 28–36 (e) of development: a) The Ki-67 antigen is expressed by individual cells of the metanephric blastema, vesicles and ampoules of the ureteral bud (x400); b) The Ki-67 antigen is most strongly expressed by cells in the surface layer of the nephrogenic zone, and a weak immunopositive reaction is observed in the apical region of the epithelial cells of the proximal tubules of the nephron and individual cells within the renal corpuscles, (x100); c) The Ki-67 antigen is expressed by individual cells within the glomeruli of immature renal corpuscles and, sporadically, cells in the tubules of the forming cortex (x200); d) On the cross-section of the medulla, a small number of Ki-67 immunopositive cells can be seen in the collecting ducts and narrow parts of Henle's loop (x400); e) A Ki-67 immunopositive reaction is observed in the epithelial cells of some distal nephron tubules and medullary rays (x200)

Discussion

Almost one-third of all developmental anomalies in young children are structural or functional abnormalities of the urinary tract. Their morphological description has been well documented for decades, but the mechanisms of their origin have not been fully explained. The reason for this is that much data on normal and abnormal nephrogenesis are derived from studies of animal models, most commonly mice. There are numerous studies done with the aim of explaining key developmental processes, primarily cell proliferation and apoptosis, their genetic basis, and control mechanisms. These studies are diverse in type, methodology, and aims, while studies on human material, embryos, and fetuses are relatively few due to ethical constraints (5, 17, 19, 20). During the last 20 years, by applying gene expression manipulation techniques, as well as experimental models with cell cultures, great progress has been made in identifying cellular and molecular mechanisms that direct normal renal morphogenesis but also provide insight into the possible pathogenesis of anomalies (21).

However, caution is needed in interpreting the results of experimental studies because it has not been established with certainty whether the deciphered developmental principles and active executory molecules are the same in the case of human kidney development. In the broadest biological sense they are, but the influence of a

spectrum of genetic and epigenetic factors (race, climate, age and maternal malnutrition, drug consumption) has been proven to impact developmental processes. Therefore, very little is still known about the cell biology of most kidney malformations (11, 14).

Kidney development is a continuous series of complex processes (induction, proliferation, differentiation, apoptosis, morphogenesis) which simultaneously, but with different intensities and in different cell types, take place in the metanephros, mostly in the nephrogenic zone, the tissue of the renal cortex. Consequently, the morphology (histological picture) of the fetal kidney changes rapidly from week to week, becoming more and more complex for analysis. It is necessary to identify different cell types involved in nephrogenesis, with the help of immunohistochemical detection of their proteins expressed at the cytoplasmic or nuclear level (22).

In this study, the expression of the immunohistochemical cell proliferation marker Ki-67 was examined concerning cell type and the developmental week of metanephrogenesis to gain a better insight into the morphological aspect, as well as the dynamics of cell proliferation as a fundamental developmental process.

During the monitored developmental period, metanephros went through all the characteristic and described developmental stages, from invasion of the ureter bud into the metanephric mesenchymal blast, through all the phases of glomerulogenesis and the gradual differentiation of nephron segments, to the thinning of the nephrogenic zone into a narrow band of tissue. In all the examined tissue samples, the proliferation marker Ki-67 was expressed in different structures of the metanephros, but with different intensities and distribution.

This study also showed that nephrogenesis occurs from the deep to the external cortex (24, 25). In the examined period, the pronounced proliferation was limited almost exclusively to the external nephrogenic cortex, the subcapsular belt of tissue which, during nephrogenesis, is where new nephrons are formed under the inductive influence of growing branches of the urethral bud. Ki-67 positive cells were observed at the tips of the branches of the urethral buds indicating the elongation and branching of the collecting duct system. These cells were also present in the condensed mesenchyme of this zone, along with the developmental forms of the renal corpuscles such as vesicles and S-forms. All together, these observations reflect the activity of the formation of new nephrons. Over time, the expression of Ki-67 decreased rapidly as the nephrons matured. This pattern of expression correlates with those of previous studies (25) of human fetal kidneys, which described that in later stages of renal development, decreased Ki-67 expression occurs with progressive glomerular maturation, while in terminal differentiated glomeruli Ki-67 expression is absent (26).

The phenomena described in this study occur in individual cells but are a necessary part of morphogenesis, a developmental process by which groups of cells acquire complex three-dimensional forms, which could be tracked by analyzing the results of this study. Examples include the serial dividing of ureteral bud branches, the formation of glomeruli and winding nephron tubules from blastemic mesenchymal cells, capillary formation by angiogenesis and vasculogenesis, and the relationship between the cortical and medullary zones. It was noticed that in the earlier stages of development, the defined cortical zone is very narrow with a wide nephrogenic zone, while the medulla is wide and the corticomedullary border is unclear. The proliferative activity in the renal medulla was visible only as the presence of individual Ki-67 positive cells in the epithelium of the collecting ducts, Henle's loops, and the interstitium. Unlike the parenchyma of the cortex, which is constantly building and expanding as a result of the formation of a generation of corpuscles arranged in pillars around Ferrein's pyramids, the medulla is relatively narrowed and necessarily and constantly remodeled so that the rapidly growing number of nephrons can drain when they become functional. This interpretation is consistent with the morphometric study (16, 27) which showed that while the number of glomeruli increased 50-fold from 15 to 40 weeks, the average volume of the cortical segment of the nephron (tubuli contorti) initially decreased and then increased. Simultaneously, there was a constant increase in the average volume of the medullary segment of the nephron (Henle's loop) so that the overall fractional volume of the renal cortex decreased.

Conclusion

The results of this study showed that cell proliferation was continuously present during metanephrogenesis. It took place with different dynamics, was more pronounced in the nephrogenic zone and renal cortex due to the dominance of cells in their structural components, but the evident developmental remodeling of these tissues indicated the need to correlate proliferation with other developmental processes, apoptosis above all.

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KI-67 MARKER ĆELIJSKE PROLIFERACIJE U HUMANOJ METANEFROGENEZI

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Bubreg obavlja više neophodnih funkcija, poput izlučivanja metaboličkog otpada, održavanja ključnih parametara homeostaze krvne plazme, učešća u regulaciji krvnog pritiska i nivoa hormona. Ove raznovrsne funkcije omogućava proces razvoja, koji je osigurao prisustvo specifičnih ćelija za obavljanje svih složenih zadataka. Organogeneza bubrega je kompleksan proces koji uključuje proliferaciju ćelija kao osnovni nužni proces. Cilj ove studije bio je da se utvrdi proliferativna aktivnost u toku faze razvoja metanefrosa na osnovu profila/izgleda prostorne i vremenske ekspresije markera ćelijske proliferacije Ki-67. Analizirani su uzorci bubrežnog tkiva 30 ljudskih fetusa gestacijske starosti od 11 do 36 nedelja. Uzorci su podeljeni u tri grupe na osnovu perioda razvoja, koji su odgovarali ranijem, srednjem ili kasnijem periodu gestacije. Rutinskom histološkom obradom dobijeni su isečci tkiva na kojima je proliferativna aktivnost ćelija (ekspresija proteina Ki-67) ispitivana imunohistohemijskom metodom, monoklonskim antitelom Ki67, i to prema protokolu proizvođača.

Ćelije pozitivne na Ki-67 karakterisale su sa različitim intenzitetom sve strukture metanefrosa. Najizraženije je bilo njihovo prisustvo u nefrogenoj zoni u ranijim nedeljama razvoja, što ukazuje na ulogu proliferacije ćelija u formiranju nefrona. Intenzitet ekspresije Ki-67 antigena postepeno je opadao u svim kortikalnim strukturama do kraja ispitivanog perioda. U meduli metanefrosa proliferacija je bila slabije izražena samo nakon 20. nedelje; bile su pozitivne na Ki-67 pojedinačne epitelne ćelije sabirnih kanala, uskih delova Henleovih petlji i intersticijuma.

Proliferacija ćelija bila je kontinuirano prisutna tokom metanefrogeneze; odvijala se različitom dinamikom, a bila je izraženija u nefrogenoj zoni i bubrežnom korteksu zbog dominacije ćelija u njihovim strukturnim komponentama. Evidentno prisutno razvojno remodelovanje tkiva bubrega ukazalo je na potrebu korelacije proliferacije sa drugim razvojnim procesima, pre svega apoptozom.

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***Ključne reči**: razvoj bubrega, metanefrogeneza, proliferacija ćelija, Ki-67*