INVESTIGATION OF COLORECTAL TUMOUR PROGRESSION BY AN EXPERIMENTAL ANIMAL MODEL

PhD Thesis Outlines

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Introduction

In developed countries, colorectal cancer (CRC), or rather its progression to metastatic disease accounts for 25% of tumour deaths. Surgical treatment of early-stage CRC provide good overall survival rates as the main oncosurgical technical criteria have been clarified over the past two decades. On the other hand, effective treatment of locally invasive (T4) tumours, as well as distant metastatic (M1) lesions has not been discovered, so far. Characterising the metastasis associated processes is therefore of crucial importance for identifying ways of earlier and more sensitive diagnosis, more defined prognosis, and possibly in the selection of patients for targeted therapies.

A variety of genes have been described and extensively investigated in the literature as key candidates in the tumorigenesis of colorectal carcinoma, including APC, Tp53, KRAS, BRAF, DCC, EGFR, SFK, TGFR2, SMAD4, etc. Alternative carcinogenesis pathways (MSI, IBD, ER) have also been identified leading to colorectal cancer, too. These experimental issues are gradually being involved in taylored, targeted treatment modalities.

Morphologic cascade of tumour progression events has been well defined (invasion, epithelial-mesenchymal transition, angiogenesis, intravasation, survival in circulation, adhaesion, extravasation, mesencymal-epithelial transition), as well. However, metastatic process seems to be a more complex, dinamically changing genetic, epigenetic and regulatory issue, so that an acceptable, simplified model of metastasis (alike adenoma-carcinoma sequence in carcinogenesis) has not yet been set up.

There are tumour- and host-associated (microenvironment-associated) conditions which need to be fulfilled at a time for the excellent performance of metastatic cascade. This kind of duality can be recognised all over the clone selection process of metastatic subclones in primary tumour (or in the enrichment of metastatic stem cells in an other, „metastatic stem cell model") which is somehow affected or even driven by microenvironmental selection factors.

One of the main results of HUGO project was that no more than 25.000 genes can be identified in human genome. Phenotypic variability largely exceeds this number, the background of which is partly given by alternative splicing phenomenon. At least 75-90% of human genes are affected by alternative splicing, which leads to a number of

structurally and functionally different protein products. Obviously alternative splicing is not a random process, it is rather strictly regulated. Transcriptional variability as a result can even modulate the dinamic regulation of tumuorigenesis and tumour progression, as well.

CD44 is one of the best representatives of antelnative splicing. Although it is as CD44 'in general' knownt to be one of the most investigated stem cell markers, more than 1000 potential mRNA isoforms of the molecule should rather make it called CD44-family. Standard CD44 contains only 1 of the 10 variant exons. It is expressed in normal tissues of epithelial, as well as haemopoetic origin. Neoplastic tissues, however, express a few isoforms (splice variants) with different assemblies of variant exons. Functional characterisation of the standard and variable exons of CD44 (and hence variable domains of CD44 protein) has been carried out recently, modulations of their behaviour by their co-expression and cooperation has not been extensively investigated, so far. Functional and prognostic value of v3 and v6 variant-containing isoforms have been mainly investigated in the literature in accordance with tumour progression (especially in colorectal cancer), however results are rather controversal and heterogenous.

Similarly, WT1 gene can be characterised by functionally different splice variants. There are at least eight identified splice variants of WT1, by now. Its splice variants represent functionally different characters. They can be transcriptinal factors (acting as oncogenes) and tumoursuppressor, too. Different splice variants of WT1 perform different functions in different phases of tumorigenesis and tumour progression. Different tissue microenvironments can cause even more controversal characters of the same molecule. Therefore, simple and exact definition of the role of WT1 would be more than difficult, it can be best described as a dinamically changing regulator element. Recently WT1 has been put on the top of the potentially targetable genes' (and gene products') list in different haematological and solid (e.g. coorectal) malignancies as it seems to play a central role in the regulation of tumour progression.

In contrast to the „potential target entity" of WT1, KRAS gene has already been proven to be one of the most effective prediction markers in targeted (anti-EGFR) oncotherapy. Activating mutation of KRAS gene can be identified in 30-50% of colorectal cancer cases. Clinical epidemiology found its mutation status to be well concordant with anti-

EGFR therapy resistance in metastatic colorectal cancer (mCRC), as KRAS is the top element of the main downstream signalling pathway of EGFR.

Aim of the study

Our main goal was to design, set up and test an experimantal animal model system, which could be appropriate and adequate to test genes contributing in metastasis of colorectal cancer.

We aimed the model system to be suitable to give answers on questions like: in which phase of the complex metastatic cascade does a gene or a group of genes carry a functional role. Furthermore we wanted the model to give a chance to distinguish between molecular biological events in primary and metastatic tumour, or even in the phases between the two endpoints (e.g. in lymph node metastases or in circulating tumour cells).

As practical testing of the animal model system we aimed to perform the further experiments and pose the next questions:

1. We aimed to examine the differences between treatment sensitivity of primary and metastatic colorectal tumour against different therapeutical agents of clinical oncology.

2. We wanted to examine the expression pattern of CD44 alternative splice variants over human colorectal tumour progression. As an experimental control gene, WT1 was used, several alternative splice variants of which performs de novo expression in colorectal cancer.

3. Finally, we aimed to investigate the clinically relevant open question, whether KRAS mutation status of primary colorectal cancer can be representative enought for the status of metatasis, and hence can act as a predictor of targeted oncotherapy.

Methods

1. Animal model

We used two different tumour implantation systems: iso- and xenograft systems.

In the *isograft* system we injected suspensions of the C26 isograft colorectal tumour cell line into adult Balb-C mice. In this system hosts are not immune to tumour grafts, therefore immunosuppression is not needed.

In the *xenograft* system, xenotransplantation of three human colorectal cancer cell lines (HT25, HT29, HCT116) was performed in adult *scid* mice. Rejection of tumour grafts was prevented by immunodeficiency of the *scid* mice.

1.1. Liver metastasis models

Cell suspensions of three gentically different human CRC cell lines and a mouse CRC line were implanted into scid and Balb/C mice as xeno- and isografts into different localisations. Implantations were carried out *orthotopically* into coecal wall and *heterotopically* into the spleen. In both groups primary tumours and distant secondaries (in the liver) were developed. Liver secondaries were real metastases in the orthotopic model, but „just" second primaries (liver colonies) in the splenic implantation group. Eight to twelve weeks after implantation autopsy samples of the tumours gave chance to investigate primary and secondary tumours at a time.

1.2. Investigation of the effect of tumour microenviroment: adult-newborn experiment

Cell suspensions of the three human CRC lines (HT29, HT25, HCT116) were implanted *subcutaneously* into *adult* and *newborn scid* mice.

None of the adult mice, but each of the newborn ones developed distant (pulmonary) metastases beside subcutaneus primary tumours.

All animal experiments have been approved by the local Animal Experimental Research Board (TUKEB83/2009).

2. Examination of therapy sensitivity of colorectal tumour cell cultures of different stage.

We developed cell cultures of solid (primary, lymph node metastasis and liver metastasis) tumours, as well as of circulating tumour cells of the Balb/C-C26 isograft

liver metastasis model. Chemosensitivity of the cell cultures originating from different tumour localisations were compared by MTT-proliferation assay.

Dose- and timedependent effect of the rouitinely used chemotherapy agents and targeted drugs (5-fluoro-uracil, leucovorine, oxaliplatine, irinotecan, cetuximab, bevacizumab, imatinib) were examined in monoterapy and combination treatment.

3. Gene expression studies:

3.1. RNA-isolation and RT-PCR

Total RNA was isolated with Trizol® from cell cultures and frozen homogenized tumour tissues. Possible DNA contamination was eliminated with TURBO DNA-free™. Reverse transcription was carried out and controlled by PCR reaction with β-actin housekeeping gene specific primers.

3.2. Qualitative PCR

3.2.1. PCR detection of CD44-fingerprint, sequencing

For investigation of the variable regions of CD44, serial PCR reactions were carried out with five different human-specific primer pairs. Primer pairs were designed the way to provide multifold overlap of the region of interest (especially exons v3 and v6).

PCR products were separated on a 3 % agarose gel and detected with Gel Doc 2000 (Bio-Rad®) after ethidium bromide staining.

Transcribed isoforms were identified by re-extraction (High Pure PCR Product Purification Kit (Roche, Mannheim)), and direct sequencing (Big Dye Terminator cycle sequencing, Applied Biosystems 3130 Genetic Analyzer), as well as next-generation sequencing (Roche 454 GS Junior).

3.2.2. Detection of WT1 alternative splicing pattern by nested-PCR

WT1 expression was examined by nested PCR. Following total RNA isolation and reverse transcription, PCR amplification from cDNA was performed in two steps. Reaction products of „inner” reaction were separated on a 4% agarose gel and visualized with Gel Doc 2000 (Bio-Rad®) after ethidium bromide staining.

3.3. Real-time PCR with specific primers of variable exons of CD44

For quantitative measurement of CD44v3 and v6 variable exons q-PCR reactions were used (SYBR® Green (Bio-Rad)) on cDNA of isolated tumour samples. Starting quantities were defined on the basis of standard five-fold dilution series carried out with control cDNA of A431 (human squamous cell carcinoma). Relative expression of the examined v3 and v6 variable exons were determined by normalizing the starting quantities to the housekeeping β-actin starting quantities from the same cDNA sample.

3.4. Detection of KRAS mutation status – RFMD (Restriction Fragment Microfuidic Based Detection)

Representative samples of Balb/C-C26 isograft system (primary colon cancer, primary spleen tumour, liver secondaries, lymph node metastases and circulating tumour cells) were obtained and primary cell cultures were induced. Total RNA was isolated from confluent cultures, then reverse transcription was performed followed by checking of the sample purity and the success of transcription by β-actin PCR.

DNA sequence containing codon 12 was amplified by sequence-specific primers. PCR product was digested with BstNI (New England BioLabs) restriction endonuclease. Semi-quantitative evaluation of the reaction was performed by Experion™ DNA 1K Analysis Kit (Bio-Rad).

Results

1. Animal model system

Success rate (metastasis formation) in isograft system (Balb/C-C26) both on orthotopic implantation and heterotopic (intrasplenic) implantation arms were 100%.

In our xenograft liver metastasis system liver metastasis formation rate was higher than 50% with each three human colorectal cell lines in *scid* mice.

The „adult-newborn" experimental system worked quite specially: each animal developed primary subcutaneous tumour, but none of the adults formed distant metastasis while every single newborn ones had macroscopic pulmonary metastases (0%-100%).

2. MTT test for analysing chemosensitivity of experimental primary and metastatic colorectal cancer against different chemotherapy agents.

A unique case of real colorectal liver metastasis animal system (Balb/C-C26) provided tumour samples of different localisations. Cell cultures were initiated from primary colon tumour, lymph node metastasis, liver metastasis and circulating tumour cells at the same time. Proliferation activites of the four cell lines were different, but each was almost perfectly exponential.

5-fluoro-uracil, oxaliplatine and imatinib monotherapy provided time-dependent inhibitory effect on the four cell cultures. Circulating tumour cell line and lymph node metastasis line were more resistant to therapy than the primary and the liver metastasis lines.

Combination of 5FU, leucovorin and oxaliplatine performed a similar time-dependent inhibitory manner, but differences between chemosensitivity among the four cell lines diasappeared.

Monotherapy effect of irinotecan was rather inconsistent, but combination therapy of 5FU, leucovorin and irinotecan was characterised by pronounced inhibitory effect on each cell line without major differences.

Finally, cetuximab and bevacizumab did not inhibit proliferation.

3. CD44 expression on colorectal cell lines and tumour samples

We identified CD44 isoforms expressed in CRC cell lines from a qualitative picture derived from five-primer-pair PCR series covering the entire length of the variable region of CD44 with overlapping sequences. By running the PCR products of the five different primer pair reactions on agarose gel always in the same order, a colorectal cancer specific alternative splice pattern (ASP) could be described.

3.1. CD44 alternative splice pattern of human CRC cell lines

CD44 ASP on each human colorectal cell line (HT29, HT25 and HCT116) was found to be identical.

3.2. Identification of colorectal specific CD44 isoforms, resolution of CD44 ASP.

Clonal sequencing by next-generation sequencing technique made it possible to identify CD44 isoforms with extremely high resolution in a tumour sample. Beside the number of variant isoforms identified by direct sequencing NGS provided several further isoforms, as well. A few further isoforms could be just estimated by calculation of product lengths. After all we managed to set up a rich roster of colorectal specific CD44 isoforms consisting of 26 well distinguishable variants, which is the most detailed list of ist kind, so far.

Additionally, we described v0 variants among CD44 isoforms, therefore we proved that in contrast to the general literary view, v1 is in fact variant in human, as well.

3.3. Colorectal specificity of CD44 ASP

We demonstrated colorectal specificity of CD44-fingerprint by comparing it to CD44 ASPs of other tumour types, i.e. MCF7 (breast cancer), HT199 (melanoma), A431 (vulvar epidermoid carcinoma), and K562 (myelogenous leukemia), respectively.

3.4. Characterisation of CD44-fingerprint over tumour progression

ASP of CD44 was found to be highly similar in colorectal cancer cells cultured in vitro as compared to orthotopic primary (colon) or heterotopic primary (spleen). Pattern of the primary tumours and the liver metastases/colonies in both models proved to be unchanged.

3.5. Quantitative change of certain exons (v3/v6) of the CD44 variable region during tumour progression of colorectal cancer

As qualitative stability does not automatically mean quantitatively unchanged expression character of certain CD44 variants, we quantitatively tested our animal model on the two (from metastasis point of view) main exons, v3 and v6.

Isograft (Balb/C-C26 model)

CD44v3 and CD44v6 expressions of primary orthotopic (colonic wall) and heterotopic (splenic implantation) C26 tumours, as well as spontaneous liver metastases and liver colonies (from splenic implantation) were compared using real-time PCR. Data indicate

that while the expression level of both variable exons were more than one magnitude larger in the liver metastases compared to primary tumours in the orthotopic implantation model, no significant alteration was detected between the spleen primary and the liver colony. Furthermore, it is remarkable that primary tumours of both systems showed similar expression levels.

Xenograft human CRC liver metastasis model

Real time PCR measurement using CD44v3 and CD44v6 exon specific primers showed three different ways of behaviour in the three colorectal cell types, although they performed similar metastatic potential. In representative samples we compared the relative CD44v3 and CD44v6 levels of the primary tumours and liver metastases or liver colonies in both systems, similarly to the isograft experiments. In case of HT29, identically to C26, we detected significantly elevated expression levels in liver metastases after orthotopic implantation compared to the primary tumour of the colonic wall, while both variable exons were expressed at the same level in the primary splenic tumour and the liver colony of the spleen-liver system. It is also interesting that primary tumours from both localisations showed similar expression levels, again.

HT25 and HCT116 showed different behaviour of expression intensity changes over metastasis.

CD44 v3 és v6 expression activity of primary heterotopic colorectal cancer in permissive and non-permissive hosts (adult-newborn model)

The same three, genetically different human colorectal cell suspensions (HT25, HT29, HCT116) were implanted into lumbar subcutaneous localisation of adult and newborn scid mice. No metastasis was formed in the adult animals, while each newborn developed distant lung metastases. CD44v3 and CD44v6 expression levels of non-metastatic adult and metastatic newborn primary tumours were measured. 2-3 fold higher v3 and v6 expression levels were measured in the newborn than in adult primary tumours.

4. WT1 gene expression in progressive human colorectal cancer

Ectopic, *de novo* expression of WT1 in colorectal cancer is an evidence of the literature. Overexpression and expression of certain splice variants of WT1 is associated with tumour progression in some tumours.

We examined WT1 expression pattern of three different human colorectal cell lines. Both the cell cultures and the primary and metastatic tumours of our two liver metastasis animal xenograft models were tested with WT1-specific nested PCR.

Compared to the pronounced stability of CD44 alternative splice pattern, WT1 presented a rather heterogenous expression profile. Both KTS+ and KTS-, as well as Zn-finger deficient splice variants appeared separately and in combination in primary and metastatic colorectal tumours. KTS- splice variants were generally present in each sample, differences were based on KTS+ and Zn-finger deficient isoform expressions.

We can underline that our study group demonstrated Zn-finger deficient WT1 isoforms in colorectal cancer first time in the literature.

5. KRAS mutation status in C26 – Balb/C metastatic colorectal isograft system

KRAS (codon 12) mutation status was examined in primary tumours, metastases and circulating tumour cells (cell cultures derived from the menioned tumours) in Balb/C-C26 isograft system. We determined wild type (wt) / mutant allele ratio of the samples.

First we demonstrated that wt/mutant ratio remains unchanged from original primary C26 cell culture to the primary tumours either in the colon or in the spleen.

We then proved that neither the lymph node nor the distant liver metastasis in real colorectal metastasis system show significant change in wt/mutant KRAS ratio from primary colon tumour. In contrary, circulating tumour cells seem to represent a higher mutant/wt rate.

Finally, after heretotopic C26 implantation into the spleen multiple colonies developed in the liver. These liver colonies differ from each other regarding mutant/wt KRAS status, but this rate is consequently higher than in the primary spleen tumour.

Conclusions

1. In summary, we managed to design, set up and test a complex animal experimental model system, which, correctly represents the process of colorectal cancer progression. The system is able to differentiate between different phases of the metastatic cascade, as well as enables examination of the interaction between the tumour and its microenvironment which influences tumour progression.

2. The most important result of our study is that we managed to prove that tumour microenvironment (host) plays a crucial role in the evolution of metastatic phenotype of primary colon tumour.

3. Most of the results of our experimental animal model system were derived regarding CD44 gene expression. While normal epithelial tissues (such as colonic mucosa) express only standard CD44 isoform, a huge variety of further (variant) isoforms appear in malignant tumours. The background of the phenomenon is alternative splicing (and its regultory system). Provided that different CD44 isoforms can represent a serie of new functions, investigation of „CD44" in general is rather questionable.

4. We demonstrated that colorectal specific CD44 isoform expression pattern does exist (ASP can be formed), which ASP is different from CD44 ASP gained the same way in other tumour types. This ASP - on the other hand - was found to be extremely stable within colorectal cancer type from cell cultures over primary tumours to the metastases. We generated and published the most detailed colorectal specific CD44 isoform expression roster by „Next-generation sequencing" technique. Qualitative stability of expression splice pattern, though, can still hide quantitative (expression intensity) changes of certain variants.

5. Regarding variant isoforms of CD44 (especially the ones carrying the functionally well-characterised v3 and v6) our experimental results indicate that they play an important role in the formation of metastatic phenotype. High summated v3/v6 co-expression levels represent a quasy-metastatic gene function in the primary tumour (at the early phase of metastatic cascade). Our results demonstrate that appearance of this „v3/v6 high" metastatic phenotype in a minority of the cells of a primary tumour

mass is sufficient to make the entire tumour metastatic. Although each colorectal cancer may „employ” metastatic v3/v6 high subclones, selective examination of the metastatic tumour cells has not yet been resolved. This means that neither quantity of „CD44” in general, nor the expression intensity of v3/v6 containing isoforms are suitable to predict metastatic behaviour of a unique cancer case, because of the summative art of our current examination techniques.

6. We further demonstrated that circulating tumour cells, which theoretically are very close to the metastatic subclone, can be isolated, cultured and examined.

7. We proved that isolated circulating tumour cells act differently to primary and metastatic tumours in terms of CD44 v3 and v6 containing isoform expression, KRAS mutation status and chemosensitivity, too. This can easily provide new perspectives to the taylored design of targeted oncothetapy and its background diagnostic research.

8. Furthermore we found animal experimental evidence, that KRAS mutant allele expressions in primary and metastatic colorectal cancer are comparable. Targeted therapy design has just presumed this fact on the basis of epidemiological, retrospective studies, so far.

9. Finally, via our xenograft animal CRC liver metasis model we demonstrated, that primary and metastatic colorectal carcinoma consequently express WT1, the gene which carries a crucial role in several neoplastic cellular functions. We managed to prove the existance of so far unpublished, „new” Zn-finger deficient isoforms beside the well-known splice variants in colorectal cancer. WT1 ASP, however, does not seem to be stable in colon tumours, which further underlines the importance of the stability of CD44 splice pattern.

Publications

Papers directly related to the dissertation:

1. Characteristics of CD44 alternative splice pattern in the course of human colorectal adenocarcinoma progression. **Balázs Bánky** - Lívia Rásó-Barnett, Tamás Barbai, József Tímár, Péter Becságh, Erzsébet Rásó. Molecular Cancer 2012, 11:83 EPUB doi:10.1186/1476-4598-11-83

2. Demonstration of a melanoma-specific CD44 alternative splicing pattern that remains qualitatively stable, but shows quantitative changes during tumour progression.Livia Raso-Barnett **- Balazs Banky**, Tamas Barbai, Peter Becsagh, Jozsef Timar, Erzsebet Raso. PLOS ONE 2013; 8 (1)| www.plosone.org

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1. Colonic obstruction caused by intra-luminal haematoma. Robert Thomas, **Banky Balazs**, Catherine Hobday, Davis W Borowski. BMJ Case Rep. 2012 Oct 9.EPUB doi: 10.1136/bcr-2012-006801.

2. Revisiting CB1 Receptor as Drug Target in Human Melanoma. István Kenessey - **Balázs Bánki**, Agnes Márk, Norbert Varga, József Tóvári, Andrea Ladányi,Erzsébet Rásó and József Tímár. Pathol Oncol Res 2012 Oct; 18(4):857-66.(Epub 24 March 2012) PMID 22447182.

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2. Comparative analysis of open, laparoscopic and single-port access reversal of Hartmann's procedure – poster. **B. Banky**, A. Banerjee, D. Garg, A. Agarwal, M.A. Tabaqchali, T.S. Gill, D.W. Borowski. ESCP Congress, Vienna, 2012. szeptember 26-28.

3. Nyílt, laparoscopos és „single-port" Hartmann-rekonstrukció összehasonlító elemzése – oral presentation. **B. Banky**, A. Banerjee, DW. Borowski, D. Garg, MA. Tabaqchali, A. Agarwal, TS. Gill. Magyar Sebész Társaság 61. Kongresszusa, Szeged, 2012. szeptember 13-15.

4. Mi történik azon lokálisan előrehaladott végbélrákos betegeinkkel, akiken neoadjuváns radio-kemoterápiát követően sebészi resectiót nem végzünk? – poster. **B. Banky**, A. Banerjee, DW. Borowski, D. Garg, MA. Tabaqchali, TS. Gill, A. Agarwal. Magyar Sebész Társaság 61. Kongresszusa, Szeged, 2012. szeptember 13-15.

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17. A gyomor GIST sebészi kezelésének taktikája. **Bánky Balázs,** Burány Ákos, Lakatos Miklós, Bányász Zsolt.- oral presentation. Magyar Sebész Társaság Kongresszusa, Budapest, 2006. szeptember 6-9.

18. A gyomor GIST sebészi kezelése. Burány Ákos, **Bánky Balázs**, Lakatos Miklós,

Bányász Zsolt.- oral presentation. Magyar Gastroenterologusok Társaságának Éves Kongresszusa, Szeged, 2006. június 17-21.

19. Endoszkópos sebészeti tevékenség Komárom-Esztergom megyében. – oral presentation. Bányász Zs., Jankovich M., Rigler A., **Bánky B.**, Herczegh Zs., Csőkör Z., Farkas Z., Lakatos M., Járay G. Magyar Sebész Társaság Endoszkópos Sebészeti Szekciójának XI. Kongresszusa, Zalakaros, 2005. november 3-5.

20. Endoszkópos sebészeti tevékenség Komárom-Esztergom Megyében. – oral presentation. Bányász Zs., Jankovich M., Rigler A., **Bánky B.**, Herczegh Zs., Csőkör Z., Farkas Z., Lakatos M., Járay G. Komárom-Esztergom Megyei Orvosnapok, Kisbér, 2005. október 28.

21. Ewing szarkóma az emlőben. – oral presentation. **Bánky B.**, Lakatos M., Járay G., Bányász Zs., Szűcs I. Komárom-Esztergom Megyei Orvosnapok, Bábolna, 2004. november 12.

22. A unique case of extrasceletal Ewing's sarcoma in the breast – oral presentation (2nd prize). **Bánky B.**, Lakatos M., Járay G., Bányász Zs., Szűcs I. Young Surgeons' English Language Case Report Congress, Győr, 2004. november 5.

23. Emlő Ewing szarkóma – oral presentation. **Bánky B.**, Lakatos M., Járay G., Bányász Zs., Szűcs I. Magyar Sebész Társaság Nyugat-Dunántúli Szekciójának Kongresszusa, Kehidakustány, 2004. október 15-16.

24. Vastragbélrák okozta bal colonfél ileus sürgősségi kezelése. – oral presentation. **Bánky B.**, Matvan K., Farkas A., Burány Á., Bányász Zs. Magyar Sebész Társaság 57. Kongresszusa, Pécs, 2004. június 16-18.

25. Óriás retroperitoneális liposzarkóma. – oral presentation. **Bánky B.**, Almási K., Farkas Z., Perneczky L., Farkas A., Bányász Zs. Magyar Sebész Társaság 57. Kongresszusa, Pécs, 2004. június 16-18.

26. A bal colonfél ileus akut sebészete – retrospektív tanulmány – oral presentation. **Bánky B.**, Matvan K., Farkas A., Burány Á., Bányász Zs. Magyar Sebész Társaság Coloproctologiai Szekciójának Kongresszusa, Eger, 2004. március 18-20.

27. Giant retroperitoneal liposarcoma. – oral presentation (honoured presentationdicséret). **Bánky B.**, Perneczky L., Farkas Z., Farkas A., Almási K. Young Surgeons' English Language Case Report Congress, Budapest, 2003. november 7. Honoured presentation

28. A bal colonfél obstrukciót okozó tumorának terápiája – oral presentation. **Bánky B.**, Bánfalvi P., Burány Á., Rettegi M., Bányász Zs. Fiatal Sebészek Fóruma, Debrecen, 2003. március 20.

29. A máj malignus daganatainak ablatív technikái. – oral presentation. **Bánky B.** Kórházi Referálás, Szent Borbála Kórház, Sebészeti Osztály, Tatabánya, 2002. december 16.

30. Multimodality treatment efforts in progressive stage-IV. colorectal cancer. **Bánky B.**, Sótonyi P., Péter M. - oral presentation. European Society of Surgeons Congress. Young Surgeons' English Language Case Report Congress, Budapest. 2002. november 28-30.

31. A májdaganatok kezelésének új eljárásai. **Bánky B.**, Kóbori L.- oral presentation. Fiatal Sebészek Fóruma, Nyíregyháza, 2002. október 25-26.