Ionizing Radiation Induced Molecular Changes in Normal Fibroblasts and Tumor Cells

Ph.D. Thesis

Boglárka Schilling-Tóth

Semmelweis University
Doctoral School for Pathological Sciences

Supervisors: Hargita Hegyesi, Ph.D,
Géza Sáfrány, M.D., D.Sc.

Opponents: Zoltán Marcsek M.D., Ph.D
Zsombor Lacza M.D., Ph.D,

Final Exam Committee Chairman: István Szilvási M.D., Ph.D, Csc.,
Members: József Lövey M.D., Ph.D,
Mónika Csóka M.D. Ph.D,

Budapest
2015
1. Introduction

Since the early days of radiation therapy our methods have become much more refined and our understanding of the underlying biology is much more detailed. We now know that the outcome after exposure is determined by the sensitivity of cells, the dose and the type of radiation. Yet, some fundamental questions remain unanswered. Currently, the effective damage after radiation can only be estimated roughly with the dose-response models generally used by radiologists. They should be regarded as an aid to the evaluation and comparison of clinical data but to be applied effectively they require a thorough understanding and need to be adapted to special cases. This is particularly important, because it is well known that different tissues show different degrees of radiosensitivity, and even individual patients are known to respond differently to radiotherapy. Also, in addition to the direct effect of therapeutic higher doses there is an increasing need to estimate the consequences of low-dose radiation, specifically the non-targeted effects (NTE) of ionizing radiation, such as the bystander effect and genomial instability. In recent years, a high number of studies have reported the existence of such non-targeted effects, where the damage is induced in cells not hit by radiation. Studying radiation response and individual genes that influence radiosensitivity has been proposed as a way to simplify prediction of clinical outcome. However, there are scarcely any special biomarkers for predicting the effect of exposure. This has resulted in a quest to find quantitative and prognostic biomarkers for characterization and prediction of these effects, and to describing the biological mechanisms behind their function. To this end not only cell death needs to be noted, but also the pathways induced by radiation and molecules influencing these pathways have to be understood. Thus, the aim of the PhD work presented here was to investigate reliable and sensitive biomarkers and to estimate radiation-induced direct and non-predictable effects.

Recently, a number of studies have noted that not only DNA but also other cell organelles are responsible for radiation damage. The effect of radiation on the cell can therefore be estimated by investigating the damage in organelles. For example, mitochondria play an important role in ionizing radiation response. Among the multitude of induced effects, such as oxidative stress response, ionizing radiation may disrupt mitochondrial functions and cause mitochondrial DNA damage. Therefore, mitochondria are sensitive markers for radiation damage. In this study we first used a new technique to quantitatively measure common deletions (CD) in the mitochondrial genome (mtDNA) to detect radiation-induced direct and
non-targeted effects. We studied normal human fibroblast cultures after low and moderate dose of IR to model normal tissue reaction to such exposures.

In a separate study, I examined the expression of the radiation response gene *Growth Differentiation Factor-15* (GDF-15) in mouse mammary carcinoma cell lines with the aim of modeling individual radiosensitivity responses. Radiation response genes are good substrates for such studies, since they are induced after exposure and influence the response of cells and tissues to radiation, both as a direct or as a non-targeted effect. I studied GDF-15 specifically, because our research group has identified this gene as a radiation response gene in a previously study, but its functional role in radiation response was still unknown. This cytokine is a member of TGF-β family, and is normally induced in inflammatory reactions and stress responses after DNA damage. Like other family members, GDF-15 is activated through TGF-β receptors, binds and forms heterodimers with TGF-βRII. Transacting with other target genes of the p53 pathway GDF-15 is involved in cell-cycle regulation, DNA damage repair and apoptosis. In addition, GDF-15 is also induced after ionizing radiation, and sensitivity to ionizing radiation was shown to be strongly associated with the radiation-induced expression of GDF15. This is in agreement with existing knowledge that elevated cytokine levels can caused by radioresistance in breast cancer and HNSCC.

Therefore, in this study I investigated normal, GDF-15 overexpressing and a GDF-15 silenced cell lines, to determine how GDF-15 expression affected the damage and radiosensitivity of the cells after ionizing radiation. I observed an influence of GDF-15 expression levels on survival, ROS release, and the extent of oxidative stress-sensitive CD mutation and on other radiation response genes, like TGF-β1 and 2 expression after radiation.
2. Aims

Aims of this study were to find simple methods, biomarkers for detecting the non-predictable effect of ionizing radiation (IR), such as individual radiosensitivity response, bystander effect, or genomial instability.

2.1. Description of the effect in normal cells by mitochondrial DNA impairment (Common deletion accumulation) after low and moderate dose of ionizing radiation.

2.1.1. Compare dose-dependent common deletion (CD) accumulation in radioresistant and sensitive fibroblasts after IR

2.1.2. Detecting CD accumulation in bystander cells.

2.1.2.1. Compare the bystander signal transmission methods with CD analysis.

2.1.2.2. Investigating the role of bystander signal sending donor and acceptor cells by CD analysis.

2.1.2.3. Examining the role of serotonin in the bystander effect.

2.1.3. Pursuing delayed accumulation of CD and development of genetic instability in radiosensitive and resistant long-term cultured fibroblasts

2.2. Investigating the role of GDF-15 gene in radiation response on mouse mammary carcinoma cell lines

2.2.1. Detecting the radiation induced GDF-15 expression

2.2.2. Compare the molecular effect of GDF-15 after radiation treatment in normal, GDF-15 overexpressing and GDF-15 silenced cell lines. Investigating the influence of GDF-15 expression level:

2.2.2.1. on proliferation,

2.2.2.2. on TGF-β1-2 gene expression changes

2.2.2.3. on common deletion accumulation in the cells

2.2.3. Investigating the effect of GDF-15 expression level on consequence of IR:

2.2.3.1. on radiation induced survival

2.2.3.2. on radiation induced TGF-β1 and 2 expression changes

2.2.3.3. on radiation induced ROS release and apoptosis

2.2.3.4. on GDF-15 level formed oxidative stress-induced CD accumulation
3. Material and methods

3.1. Cell cultures

3.1.1. Human fibroblast cell lines

Primary human fibroblast cell lines were established from skin biopsies taken from cancer patients treated with radiation therapy and from foreskin samples of children undergoing circumcision for medical indications. Immortalization the primary cell lines were established with plasmid transformation containing the human telomerase reverse transcriptase (hTERT) gene, as previously described. The cells were seeded on Dulbecco’ Modified Eagle Medium (DMeM) (Sigma-Aldrich) containing 10 % Fetal Bovine Serum (FBS, Sigma-Aldrich) and 1% Fungimycin/Streptomycin (Gibco, Grand Island, NY, USA).

3.1.2. Mouse tumor cell lines

LM2 mouse mammary carcinoma and its genetic variant cell lines were used. The general LM2 cell lines were genetically modified resulted the GDF-15 gene overexpressing (LM2-GDF15) and the GDF-15 silenced cell line (LM2-shGF15). The tumor cell cultures were maintained on 10 % FBS (Sigma-Aldrich) and appropriate antibiotics containing DMeM (Sigma-Aldrich).

3.2. Radiation therapy

Fibroblast cells were exposed to different single doses (0-0.1-2 Gy) of 60Co γ-rays (Gammatron-3; Siemens, Erlangen, Germany; dose rate, 0.0244 Gy/min or 0.059 Gy/min). Tumor cell lines were exposed to X-ray (THX-250, Siemens; dose-rate 1.46 Gy/min) with different single doses (0-2-4-6 Gy).

3.3. Colony forming assay

To measure radiation sensitivity, cells were seeded on four 100 mm culture dishes at a density of 500–1500 cells per dish, and 10 h later they were irradiated with 2 Gy. After incubation primary colonies in the dishes were fixed in methanol and stained with 1% methylene blue. Colonies consisting of more than 50 cells were scored as survivors. Quantification of plating efficacy (PE) and surviving fraction (SF) were performed as previously described.
3.4. *Semi-quantitative polymerase chain reaction*

Studying the common mitochondrial DNA deletion (CD) polymerase chain reaction (PCR) was investigated. From the samples total DNA was isolated (MasterPure™ DNA Isolation kit, Epicentre Technologies Ltd, Madison, WI, USA) and the examined sequence was amplified by PCR. The primer set (IDT Technologies, Neymark NY, USA) used for detecting CD mutated mitochondria was validated and published by Rogounovitch and colleagues, the primers were engineered outside of the deletion. The wild type primer set was designed to amplify a region of the mtDNA within the deleted portion of the genome. To analyze the quantity of total mtDNA primers Total mtDNA primer set was used amplifying a sequence region in the D-Loop. Semi-quantitative PCR amplifications contained 1 μg DNA, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂ and 100 μM of each dNTPs, 2 units of DyNAzyme DNA Polymerase (Finnzymes, Espoo, Finland) and 1.25 pM of each primer in 50 μl. After denaturation at 95 °C for 5 min, the reaction mixture was cycled 40 times at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, finally extended at 72 °C for 3 min (iCycler, Bio-Rad, Hercules, CA, USA). PCR products were analyzed on 1% agarose gel (Sigma).

3.5. *Quantitative Real-time PCR (qPCR)*

The amount of total, wild type and deleted mtDNA copies and mRNA expression alteration after single radiation exposure were measured by quantitative Real-time PCR.

3.5.1. *Measuring the mitochondrial common deletion (CD)*

3.5.1.1. On human fibroblast

The mitochondrial common deletion was investigated on human fibroblasts (CR2, BS2, S1-hTERT, F11-hTERT) with various radiosensitibility and genetic background. To investigate radiation-induced effects, cells were seeded in 25 cm² culture dishes at a density of 5×10⁵ cells/dish. After 24 h, donor cells were exposed to either radiation or sham irradiation. PCR reactions were carried out using SYBR Green PCR kit in a 25 μl reaction volume with 1 μg DNA template, 2 U Taq DNA polymerase (Maxima SYBR Green/ROX qPCR Master Mix Fermentas, Lithuania), in a Rotor-Gene RG-3000 Real Time Thermal Cycler (Corbett Life Sciences, Mortlake, Australia). After denaturation at 95 °C for 15 min, the reaction mixture was cycled 40 times at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, finally extended at 72 °C for 10 min.

For quantitative analysis ΔΔCT method was used: relative amount of CD mutated mitochondria were normalized to GAPDH/total mtDNA relative amount and compared the treated to control cells.
3.5.1.2. On mouse mammary carcinoma cells
The CD analysis was performed on mouse mammary carcinoma cells too. Similar to the human fibroblast study validated and engineered primer sets were used. Quantifying the total mtDNA amount a primer set was designed on the 12SrRNA coding region. For quantitative analysis ΔΔCT method was used: relative amount of CD mutated mitochondria were normalized to CYC1/total mtDNA relative amount and compared the treated to control cells.

3.5.1.3. Radiation-induced long term effects in fibroblasts
To investigate the persistence of mtDNA deletions after irradiation normal (F11-hTERT) and radiosensitive (S1-hTERT) fibroblasts were irradiated with low and high doses (0.1 and 2 Gy). Irradiated cells were cultivated for 70 days after exposure. Each week 3x10^5 cells were re-cultured and the rest was harvested for DNA isolation and CD analysis by qPCR.

3.5.1.4. Bystander assay
To investigate radiation-induced bystander effects, signal sending donor cells and signal receiving target cells were seeded in 25 cm^2 culture dishes at a density of 5x10^5 cells/dish. After 24 h, donor cells were exposed to either γ-radiation or sham irradiation. Investigating the bystander effect two method was used. In the first method the cells were incubated for 1 h after irradiation, after which the conditioned medium was harvested from donor cells, filtered and transferred to the recipient cells. The donor cells were re-fed with fresh medium to study radiation-induced direct effects. DNA was isolated from the recipient cells 72 h later. Secondly co-culture method where the direct radiated cell were maintained on an insert above the bystander cells and were harvested for CD analysis 72h after exposure.

The effect of serotonin level was tested by CD analysis. For the examination F11-hTERT cells were seeded on high, low and undetermined serum serotonin level containing medium.

3.5.2. Quantitative real-time PCR assay for investigating target gene expression changes
The effect of X-ray on the expression of GDF15, TGF-β1 and TGF-β2 was also measured. Total RNA was isolated with Qiagen RNeasy kit (Qiagen GmbH, Hilden, Germany). A 1 μg quantity of total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, US) according to the manufacturer’s instructions. Relative fold inductions were calculated by the ΔΔCt analysis was performed with averaged relative levels of Polγ2 and HGPRT housekeeping genes used for normalization.
3.6 WST assay
For examination of GDF-15 level formed effect on cell growth WST-1 assay (Roche Life Sciences, Penzberg, Germany) was used in the cell lines.

3.7 Apoptosis
NucViewTM 488 MitoViewTM 633 (Biotium Inc., Hayward, CA, USA) apoptosis kit was used for detection programmed cell death in the tumor cell lines.

3.8 ROS detection
CellROX® Oxidative Stress Reagents (Life Technologies, Grand Island, NY, USA) was used for examination of ROS release in the mammary carcinoma cell lines.

3.9 ELISA assay
Investigating TGF-β1 cytokine expression TGF-β1 Mouse ELISA kit (R&D Systems, Abingdon, UK) was used.
4. Results

4.1. Results on human fibroblast cultures

4.1.1. In vitro radiosensitivity study of the cell lines
To detect in vitro radiosensitivity of the cell lines colony forming assay was used. In the assay the surviving fraction after 2 Gy ionizing radiation (SF2) was used as radiosensitivity value. Based on colony forming survival two cell lines with SF2 values higher than 0.2 were considered to have normal/resistant radiosensitivity (F11-hTERT and C1) below 0.2 (S1-hTERT and B1) radiosensitive.

4.1.2. Optimization of detecting common deletion (CD)
Optimization of detecting CD was fulfilled with semi-quantitative PCR on the fibroblasts and the PCR products were analyzed on agarose gel. For quantitative real-time PCR appropriate primers and conditions were chosen.

4.1.3. Quantitative analysis of common deletion
To quantitative analysis quantitative real-time PCR (qPCR) was used in radiosensitive (S1-hTERT and B1) and radioresistant (F11-hTERT and C1) cell lines and normalized the radiated samples to the sham-irradiated control. In long term-cultures the late delayed effect of radiation was examined.

4.1.4. Common deletion increase associated to radiosensitivity
The CD level in the sensitive cell lines was significantly higher after 2 Gy radiation on the 7. day normalized to the sham irradiated control. A dose dependent accumulation was detected in the radiosensitive and radioresistant cell lines, with a significant increase after low doses, but only in the sensitive cell line (P<0.001). Interestingly in long-term cultures a sensitivity dependent higher accumulation was observed too.

4.1.5. Bystander assay

4.1.5.1. Compares of bystander signal transmission assays
There was no significant difference between „co-culture” (ByIns) and medium transfer (ByMcs) assays (P> 0.05).
4.1.5.2. Role of direct radiated donor’s radiosensitivity and radiation dose on bystander cells

Examining the role of the direct radiated cells in bystander effect no significant association was found to radiation sensitivity or dose, the fibroblasts showed individual bystander responses.

4.1.5.3. Influence of direct radiated donor’s signal transmission ability on the bystander cells

We studied the importance of signal sending donor and signal receiving target cells in the radiation-induced bystander response using F11-hTERT and S1-hTERT fibroblasts. Bystander effects were evaluated by the transfer of conditioned medium from each pair wise combination of F11-hTERT and S1-hTERT cells. F11- hTERT cells grown in conditioned medium obtained from the same cell line exhibited significant bystander effect, while the bystander response was not detectable when these cells received conditioned medium produced by S1-hTERT cells (P=0.8826). In S1-hTERT cells receiving conditioned medium from the same cell line the bystander response was absent. Interestingly, S1-hTERT cells were able to produce significant (P=0.0069) bystander response when they received conditioned medium from F11-hTERT fibroblasts. The bystander effect was associated with the direct radiated donor’s individual bystander signal transmission ability.

4.1.5.4. Effect of serotonin serum level on bystander cells

No significant association was found between serum serotonin level and increase of CD in the bystander cells.

4.1.6. Genominal instability in long-term cultures

To investigate the persistence of mtDNA deletions after irradiation normal (F11-hTERT) and radiosensitive (S1-hTERT) fibroblasts were irradiated with low and high doses (0.1 and 2 Gy). Irradiated cells were cultivated for 70 days after single dose of IR. A radiosensitivity dependent higher accumulation was observed in CD accumulation. In the resistant cell line no significant elevated CD mutation was measured after low dose (P=0.6) but after high dose (P=0.0285), despite the sensitive cell line, showed significant higher mtDNA damage even after low (P=0.0345) and high dose (P < 0.001). There was a difference in the pattern of the CD damage accumulation after exposure. In 2 Gy irradiated F11-hTERT cells the CD level increased by about 1.8-fold at day 14 after IR and remained above the control level up to day
35. In the sensitive cell line a second wave of increase of CD mutation was observed 7 weeks after irradiation.

4.2. Results on mouse mammary carcinoma cell lines

4.2.1. The LM2 model for investigation GDF-15 level effects
The RNA expression changes after IR were measured with quantitative real-time PCR (qPCR) in the unchanged LM2 cell lines. A dose dependent increase of GDF-15 expression was found after exposure confirming that GDF-15 is a radiation response gene. GDF-15 relative RNA expression was investigated in the modified cell lines normalized to the normal GDF-15 expressing LM2 cell line. A 10-fold higher significant (P<0.01) elevated level of GDF-15 RNA expression was detected in the overexpressing LM2-GDF15 cell line, whereas a 80% successful silencing was noticed in the LM2-shGDF15 silenced line (P<0.05).

4.3. Molecular effects of GDF-15 on radiation response in mouse mammary carcinoma cells

4.3.1. Effect of GDF-15 on radiosensitivity
The effect of radiation exposure was measured by colony assay in the different GDF-15 level expressing cell lines. A higher survival of the cells was noted in the overexpressing cell line. A significant lower survival was measured in the silenced LM2-shGDF15 cell line (P=0.004) even at lower doses compared to the normal LM2 and overexpressing LM2-GDF15 cell cultures.

4.3.2. Effect of GDF-15 on cell growth
Following the cell growth by WST assay over 5 days a significant higher growth were noted in the overexpressing cell line (P<0.01), whereas silencing the gene decreased significantly the growth (P=0.02) compared to normal expressing cell line.

4.3.3. TGF-β1 gene expression changes after radiation exposure
Quantitative real-time PCR (qPCR) was used for detection TGF-β1 gene expression changes 24h after IR. A dose-dependent significant (P<0.01) TGF-β1 relative expression increase could be measured after exposure. The elevation becomes significant only at 2 Gy and higher dose.
4.3.4. **Effect of GDF-15 on TGF-β1 expression**

RNA expression of TGF-β1 was measured in the GDF-15 different expressing cell lines by qPCR. A significant (P=0.01) decrease of TGF-β1 RNA expression was measured in the GDF-15 overexpressing cell line compared to normal LM2 cell culture. A low, but not significant increase could be measured in the silenced LM2-shGDF15. Investigation of TGF-β1 release was fulfilled by ELISA where a significant lower (P=0.0136) level of this cytokine could be detected in the GDF-15 gene overexpressing cell line even at the protein level.

4.3.5. **Effect of GDF-15 on radiation induced TGF-β1 expression**

No significant changes were found by qPCR after 2 Gy dose of IR. The GDF-15 level did not influence the induction of TGF-β1 expression normalized to the sham-irradiated control in the cell lines.

4.3.6. **TGF-β2 gene expression after radiation exposure**

Quantitative real time PCR (qPCR) was used for detection TGF-β2 gene expression changes 24h after IR. No significant induction could be measured at this early time point.

4.3.7. **Effect of GDF-15 on TGF-β2 gene expression**

A significant decrease of TGF-β2 (P=0.017) could be measured in the LM2-GDF15 cell line by qPCR compared to LM2 normal GDF-15 expressing control.

4.3.8. **Effect of GDF-15 on radiation induced TGF-β2 gene expression**

The GDF-15 expression level changed the radiation induced TGF-β2 gene expression. A tendentious increase could be measured in silenced cell line (P=0.08) while overexpressing the GDF-15 caused a significant decrease (P=0.0202) after 2 Gy normalized to the sham – irradiated control.

4.3.9. **Common deletion accumulation after ionizing radiation**

The previously in human fibroblast optimized radiation-sensitive biomarker CD could be used in mouse mammary carcinoma cell lines to investigate the mitochondrial damage after exposure. A dose-dependent accumulation of CD could be measured (P<0.001).

4.3.10. **Effect of GDF-15 on CD accumulation**

Role of GDF-15 on CD accumulation was examined by qPCR. A significant decrease of CD accumulation (P=0.03) was found in the overexpressing cell line (0.346±0.0088) despite a significant higher mutation occurrence (P=0.01) was noted in the silenced cell line compared to normal GDF-15 expressing LM2.
4.3.11. **Effect of GDF-15 level on radiation induced CD accumulation**

A significant increase of CD could detected in the normal LM2 (P<0.05) and silenced LM2-shGDF15 (P=0.0089) cell lines after 6 Gy normalized to sham-irradiated control, whereas no elevated level of CD could detected the accumulation could noticed in the overexpressing cell line after exposure. It is worth noting that following the increase in time the accumulation could be noticed in the silenced line even 72h after IR (P=0,041) when the CD level depended to control level in the normal GDF-15 expressing culture.

4.3.12. **Effect of GDF-15 on radiation induced apoptosis**

Accumulation of apoptotic cells tended to the dose (P=0.06) after IR in LM2 cell line. Significant increase (P=0,025) in programmed cell death could be measured only at high 6 Gy dose.

In the GDF-15 overexpressing cell line absence of apoptotic cells could be measured (P>0,05)after 6 Gy exposure despite of a significant increase could detected in normal and 3-fold higher elevation in the silenced cell line (P<0,001) compared to sham-irradiated level.

4.3.13. **Effect of GDF-15 on radiation induced ROS release**

The influence of GDF-15 level on reactive oxygen species was investigated in the mammary carcinoma cell lines. A significant elevated of acute ROS release could be measured only in the silenced LM2-shGDF15 (P=0.0074) 30 minutes after exposure which tended to normal level (P=0.0582) but was higher than in normal and overexpressing cell line even after 24h.
5. Conclusions

This study suggests that the analysis of common mtDNA deletion is a reliable and simple quantitative technique to examine low and high dose radiation-induced direct and non-targeted effects (NTE). The functional study of GDF-15 demonstrated the molecular mechanism whereby the cytokine influences radiosensitivity. This two investigated biomarker are proposed to be suitable for specification of ionizing radiation’s unpredictable effects, such as individual radiosensitivity, direct low dose and non-targeted effect.

5.1. Accumulation of common deletion was higher in the sensitive cell lines at early on time after ionizing radiation

5.1.1. CD accumulation study suggest it is a reliable method for detecting bystander effect, where

5.1.1.1. no difference was found between medium-changing and co-culture method,

5.1.1.2. the effect on bystander cells was associated to the direct radiated donor’s bystander signal sending ability not to dose or radiosensitivity

5.1.1.3. No association was found between bystander effect and serum serotonin level

5.1.2. In the long-term culture a significant accumulation was found at low dose in the sensitive cell line. Both the resistant and sensitive cell line showed elevated level of CD after 2 Gy moderate dose, but only in the sensitive cell line could be measured a secondly increase in CD amount 7 weeks after exposure.

5.2. Role of GDF-15 cytokine was investigated in mammary carcinoma cell line and its effect on radiation response.

5.2.1. GDF-15 expression showed dose-dependent increase after exposure

5.2.2. An effect of GDF-15 expression level could be measured

5.2.2.1. on growth, the cells grew rapid at higher GDF-15 level,
5.2.2.2. on TGF-β1 and TGF-β2 expression, high GDF-15 level inhibited their expression in contrast low GDF-15 level caused expression increase

5.2.2.3. on common deletion accumulation, high GDF-15 level caused CD decreases, a high CD amount was noted at low GDF-15 level

5.2.3. GDF-15 expression influenced the effect of radiation damage on the cells.

5.2.3.1. Survival after IR was GDF-15 level dependent. High GDF-15 level inhibited cell death in contrast to low level which caused radiosensitivity.

5.2.3.2. GDF-15 level influenced the radiation induced TGF-β2 expression

5.2.3.3. The ROS release, apoptosis and oxidative stress-sensitive CD accumulation after exposure associated to GDF-15 expression level in the cells.
6. List of original articles

6.1. Journal papers


6.2. Book chapters


6.3. Conference abstracts


